Neuronal K-Cl Cotransporter: Transcriptional Mechanisms of KCC2 Gene Regulation

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"It is a good principle in science not to believe any 'fact'---however well attested---until it fits into some accepted frame of reference. Occasionally, of course, an observation can shatter the frame and force the construction of a new one..."

Arthur C. Clarke (2061: Odyssey Three)

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ABBREVIATIONS

aa	amino acid(s)
BDNF	brain-derived neurotrophic factor
bp	base pair(s)
CCC	cation-chloride cotransporter
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CIP1	cation-chloride cotransporter - interacting protein 1
CNS	central nervous system
coIP	co-immunoprecipitation
div	day *in vitro*
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
E	embryonic day
Egr	early growth response
EMSA	electrophoretic mobility shift assay
EST	expression sequence tag
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GC	granular cell
GFP	green fluorescent protein
HCV	hepatitis C virus
HEK	human embryonic kidney
ICC	immunocytochemistry
IHC	immunohistochemistry
IPL	inner plexiform layer
ir	immunoreactivity
kb	kilobase pairs
KCC	K^+-Cl^- cotransporter
KO	knockout
LSO	lateral superior olive
MHb	medial habenular nucleus
mRNA	messenger ribonucleic acid
NCC	Na^+-Cl^- cotransporter
NEM	N-ethylmaleimide
NKCC	Na^+-K^+-$2Cl^-$ cotransporter
NRSE	neuron-restrictive silencing element
NRSF	neuron-restrictive silencing factor
nRt	reticular thalamic nucleus
OSR1	oxidative stress response-1
P	postnatal day
PC	Purkinje cell
PCL	Purkinje cell layer

PCR	polymerase chain reaction
PFA	paraformaldehyde
PNS	peripheral nervous system
PVN	paraventricular nucleus
RACE	rapid amplification of cDNA ends
REST	restrictive element-1 silencing factor
RNA	ribonucleic acid
RPA	ribonuclease protection assay
RT-PCR	reverse transcription polymerase chain reaction
SCN	suprachiasmatic nucleus
siRNA	small interfering ribonucleic acid
SLC	solute carrier
SON	supraoptic nucleus
SPAK	Ste20-related proline-alanine-rich kinase
TF	transcription factor
TM	transmembrane
TrkB	tropomyosin receptor kinase B
TSS	transcription start site
USF	upstream stimulatory factor
UTR	untranslated region
VP	vasopressin

ORIGINAL PUBLICATIONS

This thesis is based on the following publications, herein referred to by their Roman numerals (**I-VI**), and on some unpublished results:

I. **Uvarov P***, Ludwig A*, Markkanen M, Pruunsild P, Kaila K, Delpire E, Timmusk T, Rivera C, Airaksinen MS. A novel N-terminal isoform of the neuron-specific K-Cl cotransporter KCC2. *J Biol Chem*. 2007 Oct 19;282(42):30570-6. (personal input - 60%)

II. **Uvarov P**, Ludwig A, Markkanen M, Soni S, Hübner CA, Rivera C, Airaksinen MS. Coexpression and heteromerization of two neuronal K-Cl cotransporter isoforms in neonatal brain. *J Biol Chem.* 2009 May 15; 284(20):13696-704. (personal input - 60%)

III. **Uvarov P**, Pruunsild P, Timmusk T, Airaksinen MS. Neuronal K+/Cl- co-transporter (KCC2) transgenes lacking neurone restrictive silencer element recapitulate CNS neurone-specific expression and developmental up-regulation of endogenous KCC2 gene. *J Neurochem.* 2005 Nov; 95(4):1144–55. (personal input - 90%)

IV. **Uvarov P**, Ludwig A, Markkanen M, Rivera C, Airaksinen MS. Upregulation of the neuron-specific K+/Cl- cotransporter expression by transcription factor early growth response 4. *J Neurosci*. 2006 Dec 27;26(52): 13463–73. (personal input - 70%)

V. Markkanen M*, **Uvarov P***, Airaksinen MS. Role of upstream stimulating factors in the transcriptional regulation of the neuron-specific K-Cl cotransporter KCC2. *Brain Res*. 2008 Oct 21;1236: 8-15. (personal input - 35%)

VI. Ludwig A*, **Uvarov P***, Soni S, Thomas-Crusels J, Airaksinen MS, and Rivera C. Egr4 mediates BDNF induction of KCC2 transcription. *Manuscript is under submission.* (personal input - 30%)

*****equal contribution

ABSTRACT

K^+-Cl^- cotransporter 2 (KCC2) maintains a low intracellular Cl^- concentration required for fast hyperpolarizing responses of neurons to classical inhibitory neurotransmitters γ-aminobutyric acid (GABA) and glycine. Decreased Cl^- extrusion observed in genetically modified KCC2-deficient mice leads to depolarizing GABA responses, impaired brain inhibition, and as a consequence to epileptic seizures. Identification of mechanisms regulating activity of the *SLC12A5* gene, which encodes the KCC2 cotransporter, in normal and pathological conditions is, thus, of extreme importance.

Multiple reports have previously elucidated in details a spatio-temporal pattern of KCC2 expression. Among the characteristic features are an exclusive neuronal specificity, a dramatic upregulation during embryonic and early postnatal development, and a significant downregulation by neuronal trauma. Numerous studies confirmed these expressional features, however transcriptional mechanisms predetermining the *SLC12A5* gene behaviour are still unknown. The aim of the presented thesis is to recognize such transcriptional mechanisms and, on their basis, to create a transcriptional model that would explain the established *SLC12A5* gene behaviour.

Up to recently, only one KCC2 transcript has been thought to exist. A particular novelty of the presented work is the identification of two *SLC12A5* gene promoters (*SLC12A5-1a* and *SLC12A5-1b*) that produce at least two KCC2 isoforms (KCC2a and KCC2b) differing by their N-terminal parts. Even though a functional $^{86}Rb^+$ assay reveals no significant difference between transport activities of the isoforms, consensus sites for several protein kinases, found in KCC2a but not in KCC2b, imply a distinct kinetic regulation.

As a logical continuation, the current work presents a detailed analysis of the KCC2a and KCC2b expression patterns. This analysis shows an exclusively neuron-specific pattern and similar expression levels for both isoforms during embryonic and neonatal development in rodents. During subsequent postnatal development, the KCC2b expression dramatically increases, while KCC2a expression, depending on central nervous system (CNS) area, either remains at the same level or moderately decreases.

In an attempt to explain both the neuronal specificity and the distinct expressional kinetics of the KCC2a and KCC2b isoforms during postnatal development, the corresponding *SLC12A5-1a* and *SLC12A5-1b* promoters have been subjected to a comprehensive bioinformatical analysis. Binding sites of several transcription factors (TFs), conserved in the mammalian *SLC12A5* gene orthologs, have been identified that might shed light on the observed behaviour of the *SLC12A5* gene. Possible roles of these TFs in the regulating of the *SLC12A5* gene expression have been elucidated in subsequent experiments and are discussed in the current thesis.

1 REVIEW OF THE LITERATURE

1.1 CATION-CHLORIDE COTRANSPORTERS

K^+-Cl^- Cotransporter **2 (**KCC2) is a large (~140 kDa) membrane protein mediating electroneutral cotransport of K^+ and Cl^- ions across plasmalemma. KCC2 belongs to a family of cation-chloride cotransporters (CCC), which embraces nine members (Gamba, 2005). CCC proteins are encoded by corresponding genes constituting a solute carrier 12 (*SLC12*) gene family (Table 1). Twelve-transmembrane (TM) structure has been predicted by Kyte-Doolittle hydropathy analysis (Kyte & Doolittle, 1982) for all CCC members except CCC9 (Fig. 1), but the experimental confirmation of this fact has been performed so far only for one CCC member - Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) (Gerelsaikhan & Turner, 2000;Gerelsaikhan *et al.*, 2006). CCC proteins are secondary active cotransporters: they derive energy for Cl^- cotransport from K^+ or Na^+ gradients, which are pre-established by the Na^+/K^+-ATPase.

The CCC family is subdivided into three branches (Fig. 2). First branch includes Na^+-dependent K^+-Cl^- cotransporters NKCC1 and NKCC2, as well as a Na^+-dependent Cl^- cotransporter NCC. These proteins mediate electro-neutral Na^+-driven Cl^- transport, regardless of their usage of K^+, and share ~55% homology in amino acid sequences. Second branch includes four K^+-dependent Cl^- cotransporters KCC1, KCC2, KCC3, and KCC4, which mediate electroneutral K^+-coupled Cl^- transport and share ~70% identity. These two aforementioned CCC branches are only about 25% homologous between each other. The third CCC branch is represented by two orphan CCC members - cation chloride cotransporter 9 (CCC9) and CCC-interacting protein 1 (CIP1), which demonstrate only ~20-25% identity with other CCCs (Gamba, 2005).

1.2 DIVERSITY OF CCC PROTEINS

Table 1 and Figure 2 do not fully reflect the real diversity of the CCC proteins; the presented situation is oversimplified because of multiple splice variants identified for the CCC members. It is well known that usage of alternative splicing plays an important role in generating complexity required for the highly elaborated molecular systems. More than 50% of human genes are regulated by putative alternative promoters, resulting in a wide variety of transcripts obtained by this mechanism (Kimura *et al.*, 2006). Moreover, about 95% of human genes can generate differentially spliced transcripts (Wang *et al.*, 2008;Pan *et al.*, 2008). The proteins obtained in a result of alternative splicing may possess different expression patterns and demonstrate different tertiary structures, phosphorylation patterns, and/or subcellular localizations. All this substantially increases multiformity of proteins and provides a molecular mechanism for fine tuning of gene function (Landry *et al.*, 2003). As an example, thousands of neurexin protein isoforms are generated from only three neurexin genes by usage of alternative promoters and alternative splicing (Missler & Sudhof, 1998).

Table 1. Members of the CCC family

CCC member	Gene name	Transported ions	Full length/ N-terminal, aa	Cloning
NKCC2	*SLC12A1*	Na+, K+, Cl-	1099/178	(Payne & Forbush, III, 1994;Gamba *et al.*, 1994)
NKCC1	*SLC12A2*	Na+, K+, Cl-	1191/262	(Xu *et al.*, 1994;Delpire *et al.*, 1994)
NCC	*SLC12A3*	Na+, Cl-	1023/143	(Gamba *et al.*, 1993)
KCC1	*SLC12A4*	K+, Cl-	1085/123	(Gillen *et al.*, 1996)
KCC2	***SLC12A5***	**K+, Cl-**	**1116/104**	**(Payne *et al.*, 1996)**
KCC3	*SLC12A6*	K+, Cl-	1150/190	(Hiki *et al.*, 1999;Mount *et al.*, 1999b;Race *et al.*, 1999)
KCC4	*SLC12A7*	K+, Cl-	1083/123	(Mount *et al.*, 1999b)
CCC9	*SLC12A8*	unknown	714/44	(Hewett *et al.*, 2002)
CIP	*SLC12A9*	unknown	915/44	(Caron *et al.*, 2000)

1.2.1 N(K)CC branch

1.2.1.1 NKCC2 (SLC12A1 gene)

Several splice isoforms exhibiting different localization and ion affinities were described for the Na^+-K^+-$2Cl^-$ cotransporter 2 (NKCC2). At least six NKCC2 isoforms exist in mouse kidney due to combination of two independent splicing events for the *SLC12A1* gene (Mount *et al.*, 1999a). First splicing generates A, B, and F variants as a result of alternative usage of corresponding cassette 4^{th} exons A, B, and F, which encode a relatively small 32-amino acids (aa) fragment carrying a part of the second TM segment (TM2) and the whole intracellular loop between TM2 and TM3 (Payne & Forbush, III, 1994; Igarashi *et al.*, 1995;Simon *et al.*, 1996a;Yang *et al.*, 1996). Out of 32 amino acid positions, 20 are identical in A/B/F splice variants and

only 3 are completely distinct. Most of the sequence difference is observed in the part corresponding to the TM2, but not to the intracellular loop. These NKCC2 variants are differentially distributed in kidney along the thick ascending limb of Henle (TALH): B- in cortex, F- in medulla, and A- equally in cortex and medulla (Payne & Forbush, III, 1994). In addition, these splice variants demonstrate different ion affinities (B>A>F), corresponding to the diverse Na^+ and Cl^- concentrations present along the TALH (Plata *et al.*, 2002). In an attempt to explain the structural properties defining the ion affinities of the NKCC2 protein, Gagnon and coworkers suggested that the intracellular loop between TM2 and TM3 segments contributes mainly to the Cl^- affinity (Gagnon *et al.*, 2004). In addition, Isenring and coauthors suggested that amino acid residues within the TM2 of human NKCC1 protein participate in the cation binding (Isenring *et al.*, 1998). Surprisingly, but the suggested residues are well conserved in the B/F/A isoforms of NKCC2, thus it is not clear how they could mediate the observed difference in the ion affinities of the A/B/F isoforms.

Not only binding affinities, but also transport capacities were reported to be different for the NKCC2-A/B/F variants (A>B>F) (Plata *et al.*, 2002). By using a functional $^{86}Rb^+$ assay the authors demonstrated an increased transport activity for NKCC2-A compared to other isoforms: NKCC2A-100%, NKCC2B-68%, and NKCC2F-62%. On the other hand, green fluorescent protein (GFP)-NKCC2-A/B/F fusion products have also demonstrated a different degree of incorporation into plasma membrane: NKCC2A-100%, NKCC2B-89%, and NKCC2F-64%. Thus, the different transport capacities of the A/B/F variants observed by Plata et al. (Plata *et al.*, 2002) might be partially explained by different incorporation of the transporters into plasma membrane, rather than the different kinetic regulation.

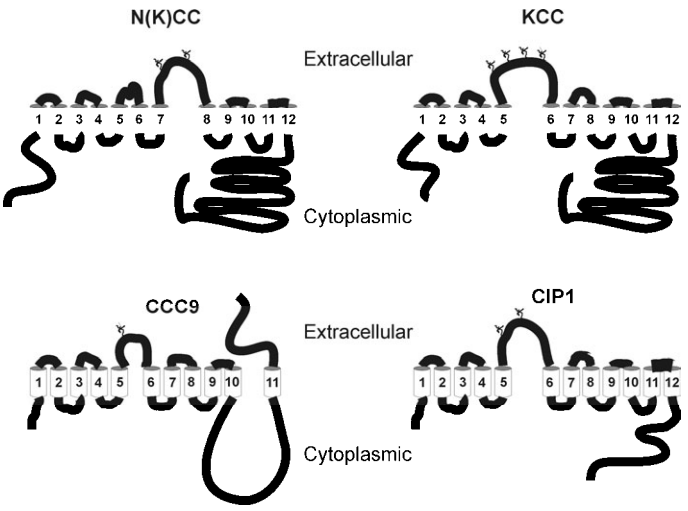

Figure 1. Domain structure of the CCC proteins. Twelve transmembrane segments were predicted for all CCC members except CCC9 (Gamba, 2005). This prediction has so far been experimentally confirmed only for the NKCC1 protein. A large extracellular loop, containing up to 4 putative glycosylation sites, was predicted in each of the CCC members.

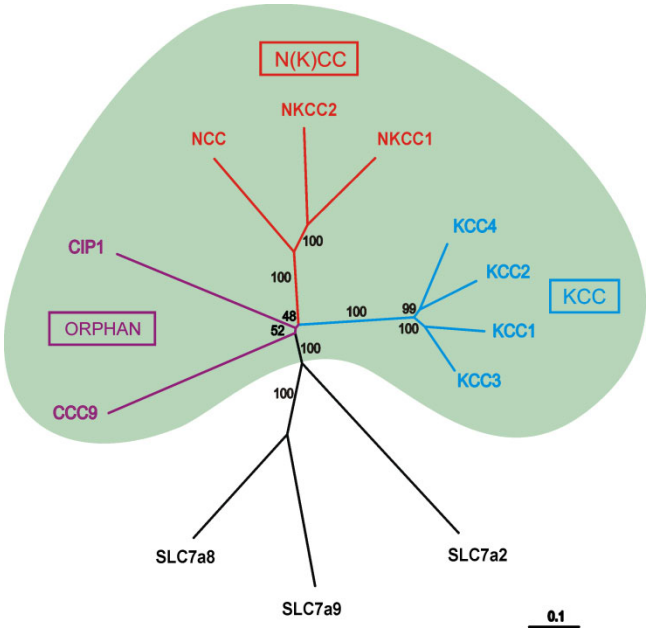

Figure 2. Phylogenetic tree of the CCC family. The CCC family (shaded in green) is currently subdivided into three branches representing the N(K)CC, KCC, and orphan members. Outgroup is represented by three members of the *SLC7* family. Analysis of the phylogenetic tree was performed using the Clustalw Tree program (Biology Workbench). Bootstrap values (a percentage of how often each branch is presented in all re-sampled trees) are indicated near the branches. Branch length corresponds to an average number of amino acid substitutions per site in the aligned sequences. The scale bar corresponds to 0.1 substitutions per site.

A second splicing event, which significantly shortens the C-terminal domain of the NKCC2 protein, was observed only in mouse kidney (Mount *et al.*, 1999a). Utilization of the extra polyadenylation site in intron-16 results in a truncation of the C-terminal domain by last 383 aa and in the appearance of a new 55-aa fragment. This short NKCC2-S variant retains an N-terminal domain and all twelve TM segments identical with the full length NKCC2-L isoform, but has a significantly shorter C-terminal part. Functionally, this C-terminally truncated NKCC2-S protein exhibits two, in a way, opposite activities. Under normal osmolarity, the protein manifests no inherent transport activity and plays even a dominant negative role by inhibiting transport activity of the long NKCC2-L isoform (Plata *et al.*, 1999). However, under hypotonic conditions, NKCC2-S gains properties of the loop diuretic-sensitive, K^+-independent Na^+-Cl^- cotransporter (Plata *et al.*, 2001). Conversion from the dominant negative molecule into the active cotransporter upon hypoosmotic shock is accompanied by active translocation of the NKCC2-S protein from cytosol to plasma membrane (Plata *et al.*, 2001). Interestingly, both the dominant negative and the transporter activities are inhibited by cAMP.

1.2.1.2 NKCC1 (SLC12A2 gene)

In contrast to NKCC2, only two NKCC1 splice variants exist, which differ by the presence or absence of the 16-aa fragment in the distal C-terminal part (Randall *et al.*, 1997). These two NKCC1 isoforms arise in a result of alternative splicing of the 48-base pairs (bp) long exon-21 in the *SLC12A2* gene. The two splice isoforms are differentially expressed in human tissues: NKCC1a is preferentially expressed in placenta, spleen, lung, heart, stomach, and salivary gland, while NKCC1b is a major isoform in brain; equal expressions of the two isoforms are

detected in muscle, liver, and kidney (Vibat *et al.*, 2001). The 16-aa fragment absent in the NKCC1b contains a putative protein kinase A (PKA) phosphorylation site, thus different kinetic regulation of the isoforms cannot be excluded.

Two complementary deoxyribonucleic acids (cDNAs) encoding slightly different variants of the NKCC1 protein in *A.anguilla* (European eel) were observed (Cutler & Cramb, 2002). Northern blot analysis revealed a ~13-kilobase pairs (kb) NKCC1a transcript, which is expressed broadly and a ~ 6-kb NKCC1b transcript, which is brain specific. NKCC1a and NKCC1b proteins share about 80% identity (Cutler & Cramb, 2002) and, most probably, have originated in a result of genome duplication that occurred during evolution of the ray-finned fish lineage (Van de Peer, 2004). Consistent with this, available complete fish genomes of *T.nigroviridis*, *T.rubripes*, *O.latipes*, *D.rerio* and *G.aculeatus* contain two genes encoding the NKCC1 protein (ensembl.org). Interestingly, ENSEMBL analysis of the aforementioned fish genomes suggests that, in addition to NKCC1, only two other CCC members, namely KCC2 and KCC4, have their genes duplicated in the fish.

1.2.1.3 NCC (SLC12A3 gene)

Rat Na^+-Cl^- cotransporter (NCC), which is expressed only in kidney, has two alternative variants exhibiting identical open reading frames (ORFs), but differing in their 3'-untranslated regions (UTRs) (Gamba *et al.*, 1994). Since messenger ribonucleic acid (mRNA) stability motifs are often located in 3'-UTRs (Shyu *et al.*, 2008), it is possible that these mRNA isoforms have different half life.

Two variants of human NCC protein, differing only by a 9-aa sequence (Simon *et al.*, 1996b;Mastroianni *et al.*, 1996), arise in a result of alternative usage of a splice donor site in exon-20, that leads to incorporation of 27 additional nucleotides into one of these NCC transcripts (Shao *et al.*, 2008). Of note, the resultant 9-aa peptide contains a putative PKA phosphorylation site (Gamba, 2005), that might also result in exceptional regulation of the human NCC ortholog. The sequence corresponding to exon-20 in human is also present in rabbit NCC, but absent from rat and mouse orthologs.

Northern blot identified two NCC mRNA variants in flatfish *P.americanus*: a major ~3.7-kb transcript in the urinary bladder and a minor ~3.0-kb transcript in several other tissues with an expression level ~100 times lower (Gamba *et al.*, 1993). The short NCC isoform is the result of alternative splicing, during which the whole N-terminal cytoplasmic domain and first three TM segments are lost (totally 229 aa), that most probably results in a non-functional NCC variant.

1.2.2 KCC branch

1.2.2.1 KCC1 (SLC12A4 gene)

Northern blot analysis of multiple rat tissues has detected at least three different KCC1 transcripts: widespread major ~3.8-kb and minor ~4.4-kb species, as well as a ~2.4-kb transcript expressed only in brain (Gillen *et al.*, 1996). In addition, both N- and C- terminal heterogeneity have been described for the KCC1, thus substantially increasing the protein multiplicity. N-terminal heterogeneity is represented by two promoters as well as by four alternative first exons (1, 1a, 1b, and 1c), which are spliced to exon-2 (Crable *et al.*, 2005). Two promoters are situated ~2 kb apart from each other and control

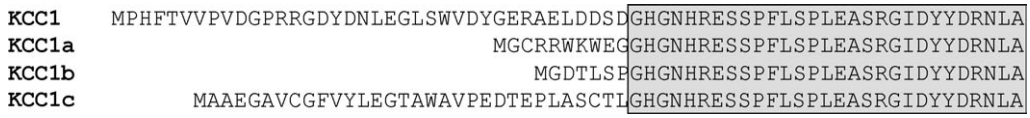

Figure 3. N-terminal heterogeneity of KCC1. Four N-terminally distinct KCC1 isoforms are generated by splicing of four alternative 1st exons to exon-2 of the *SLC12A4* gene. Common parts of the KCC1 isoforms encoded by exon-2 are shaded in grey.

expression of the KCC1 transcripts containing either exon-1 or exon-1b as a starting exon (Crable *et al.*, 2005). Exons 1 and 1b encode 39 and 7 unique amino acids, respectively (Fig. 3). These splice isoforms are known to be differentially expressed in sickle compared to normal reticulocytes (Crable *et al.*, 2005). Human expression sequence tag (EST) clones containing exons 1a and 1c spliced to exon-2 have also been found, but no further characterization of the corresponding transcripts exists so far (Fig. 3).

Analysis of the KCC1 C-terminal splice isoforms also revealed a quite complicated picture. In addition to the full-length major KCC1 isoform, there are several C-terminal isoforms that are formed in a result of inclusion of introns -21, -22, and -23 of the *SLC12A4* gene (Pellegrino *et al.*, 1998; Adragna *et al.*, 2004; Crable *et al.*, 2005). Insertion of either intron-22 or intron-23, both of which contain premature stop codons, results in truncation of the full-length KCC1 protein from the C-terminus by 74 or 17 amino acids, respectively (Pellegrino *et al.*, 1998;Adragna *et al.*, 2004). On the contrary, failure to splice intron-21 (210 bp long) leads to inclusion of an additional 70-aa fragment; intermediate products have also been described that are formed in a result of incomplete splicing of both intron-21 and intron-22 (Crable *et al.*, 2005). The physiological role of these different KCC1 splice variants is unknown, however the last 18 C-terminal amino acids, highly conserved in all CCC members, are known to be essential for interaction of the KCC3 protein with a brain-type creatine kinase (Salin-Cantegrel *et al.*, 2008).

1.2.2.2 KCC2 (SLC12A5 gene)

Until recently only one isoform of the KCC2 protein was known (Payne *et al.*, 1996) and initial efforts failed to detect additional splicing isoforms (Blaesse *et al.*, 2006). However, a new isoform of the KCC2 protein, named KCC2a, has recently been characterized by our group (I). This KCC2 variant is a result of alternative splicing of a new exon-1a, transcribed by a novel promoter, to exon-2, which is common for both isoforms. The originally characterized KCC2 transcript (Payne *et al.*, 1996), which contains exon-1b instead of exon-1a, has been renamed as KCC2b. Exons 1a and 1b encode 40 and 17 amino acids, respectively. Distinct phosphorylation patterns, present in the KCC2a and KCC2b variants, imply different regulation of these isoforms (II).

1.2.2.3 KCC3 (SLC12A6 gene)

Similar to KCC1, extensive N-terminal heterogeneity is observed for the KCC3 protein. Initial cloning of KCC3 simultaneously by three groups (Mount *et*

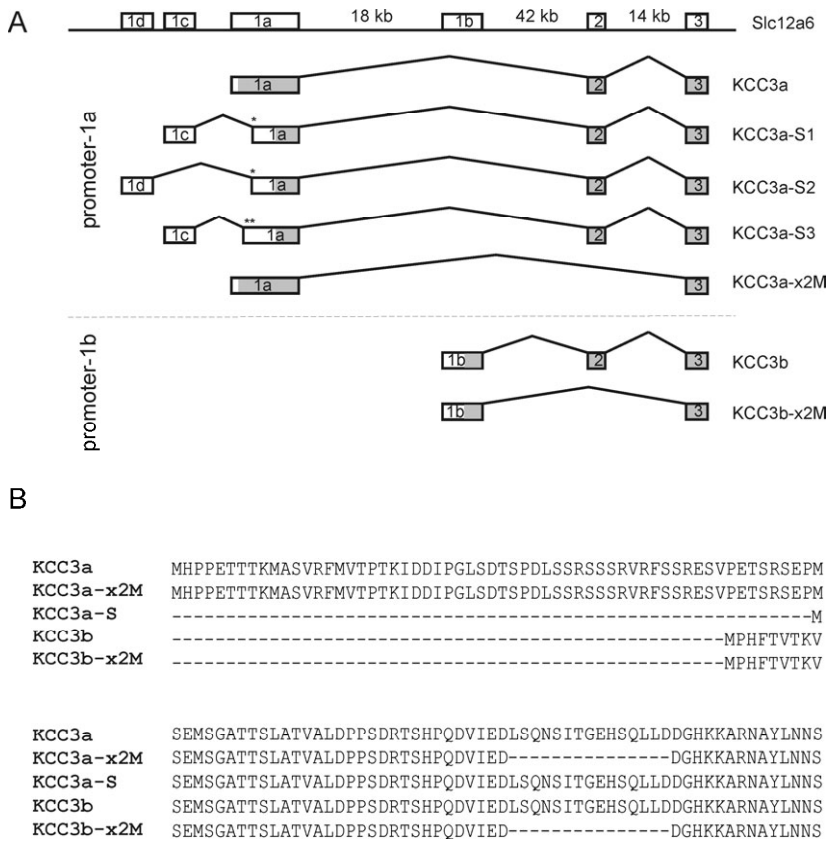

Figure 4. N-terminal heterogeneity of KCC3. (**A**) Seven N-terminally heterogeneous KCC3 transcripts are generated by two alternative promoters (1a and 1b), four alternative first exons (1a, 1b, 1c, 1d), three alternative acceptor splice sites within flanking exon-1a (marked by * and **), and one alternatively spliced exon-2 of the *SLC12A6* gene. (**B**) Since exons 1c and 1d are noncoding, there are only five KCC3 protein variants differing in their N-termini. Designations S and x2M in the names of the KCC3a isoforms reflect the N-terminal truncations (result of the alternative splice sites) and the alternative splicing of the exon-2, correspondently. Modified from Mercado *et al*., 2005.

al., 1999b; Hiki *et al.*, 1999; Race *et al.*, 1999) revealed existence of two KCC3 variants with different N-terminal parts. A more recent study has described totally seven N-terminally heterogeneous KCC3 transcripts (Fig. 4A) generated by the *SLC12A6* gene as a result of two alternative promoters (1a and 1b), four alternative first exons (1a, 1b, 1c, 1d), three alternative acceptor splice sites within flanking exon-1a (marked by * and ** in Fig. 4A), and one alternatively spliced exon-2 (Mercado *et al.*, 2005). Since exons 1c and 1d are noncoding, there are only five KCC3 protein variants differing in their N-terminal sequences (Fig. 4B). A very distinct pattern of the putative phosphorylation sites was revealed for the KCC3 variants, implying complex kinetic regulation. All KCC3

isoforms were found to be functional, but they demonstrated different transport activities in oocytes (Mercado *et al.*, 2005). For example, while all KCC3a-derived variants together with the full-length KCC3b isoform showed a very similar $^{86}Rb^{+}$ uptake in a broad range of extracellular osmolarity, the truncated KCC3b-x2M protein exhibited more than 10-fold lower activity. The rate of activation upon osmotic shock was also different for the KCC3 variants: the full-length KCC3a and KCC3b isoforms were activated ~50% slower compared to the truncated KCC3a-x2M and KCC3-S isoforms. It is interesting that exon-2 is alternatively spliced in neuronal and glial cells: expression of the KCC3a-x2M isoform has been detected in neurons but not in glia, while the full-length KCC3a isoform demonstrated the opposite expression pattern (Le Rouzic *et al.*, 2006).

SLC12A6 gene mutations, associated with hereditary motor and sensory neuropathy with agenesis of the corpus callosum (HMSN/ACC) disease (Dupre *et al.*, 2003), often lead to C-terminal truncations and, as a consequence, to the inactivation of the KCC3 protein (Howard *et al.*, 2002;Uyanik *et al.*, 2006;Salin-Cantegrel *et al.*, 2007). All these truncated isoforms lack the 18 most C-terminal amino acids, required for interaction of the KCC3 (Salin-Cantegrel *et al.*, 2008) and probably KCC2 (Inoue *et al.*, 2004) proteins with a brain-type creatine kinase.

1.2.2.4 KCC4 (SLC12A7 gene)

Two isoforms of the KCC4 protein have been reported so far. The first was identified by Mount and coworkers in their original study describing the cloning and characterization of KCC4 (Mount *et al.*, 1999b). The second isoform, which differs only by five additional amino acids inserted into the C-terminal part of KCC4 protein, was detected in olfactory epithelium but not in kidney (Nickell *et al.*, 2007). Encoded by a novel short (15 bp) exon and situated in between previously known exons 22 and 23, the same 5-aa peptide is present in the KCC2 protein sequence, thus suggesting a similar putative alternative splicing for the *SLC12A5* gene as well.

Recently, in an attempt to identify new putative promoters and/or upstream exons for the *SLC12A7* gene, encoding the KCC4 protein, I have identified an EST clone (BB662276, mouse P16 heart) containing a previously unknown exon (named exon-1a) spliced to exons 2, 3, and 4 of the *SLC12A7* gene (unpublished observation). This new isoform has been named KCC4a in contrast to the previously characterized KCC4b isoform. Exon-1a encodes for a novel 70-aa N-terminal KCC4a fragment instead of a 41-aa peptide encoded by exon-1b in the KCC4b variant. A CpG island is found in the close proximity of exon-1a, thus indicating the presence of a putative promoter region. Of special interest is a consensus sequence for a Ste20-related proline alanine rich kinase (SPAK) binding site detected in the N-terminus of the KCC4a, but not of the KCC4b variant. The presence of SPAK binding sites in all members of N(K)CC and KCC branches except KCC1 (Fig. 5), presumably points at a special role of the SPAK kinase in regulation of the CCC proteins.

1.2.3 Orphan CCC members

1.2.3.1 CCC9 (SLC12A8 gene)

Multiple alternatively spliced transcripts for the *SLC12A8* gene have been reported

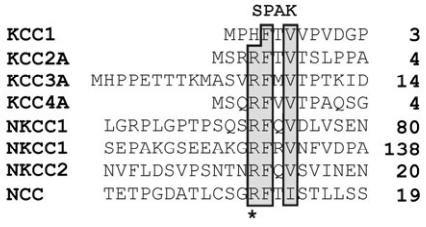

Figure 5. Conservation of the SPAK binding consensus among the CCC proteins. The SPAK binding consensus is found in all N(K)CC and KCC members except KCC1. The NKCC1 protein has two SPAK binding sites separated by 54 aa. Asterisk (*) marks +1 position of the consensus, while numbers on the right indicate the location of the consensuses in the CCC proteins. Note that the SPAK binding consensuses are situated exclusively in the N-terminal parts.

(Hewett *et al.*, 2002). The authors identified at least 13 exons that are spliced alternatively to produce totally six CCC9 isoforms. Importantly, the ultimate number of exons for the *SLC12A8* gene is definitely more than thirteen, because Hewett and coauthors in addition amplified a 456-bp 5'-fragment of CCC9 cDNA that they have not mapped properly. In agreement with this, splicing of 19 exons is known to form a 4079-bp long mRNA (XM_516709) encoding one of the putative CCC9 protein isoforms in *P.troglodytes*.

1.2.3.2 CIP1 (SLC12A9 gene)

No isoforms for the CIP1 protein are currently known (Caron *et al.*, 2000).

1.2.3.3 CIP1 and CCC9: should they belong to the CCC family?

Although CIP1 possesses a secondary structure with twelve TM segments common for CCC proteins, it has extremely low if any $Na^+/K^+/Cl^-$ transport activity (Caron *et al.*, 2000). One possibility is that CIP1 protein requires other partners to reveal its transport activity- as in the case of the glycoprotein-associated amino acid transporters (gpaAT) family members (*SLC7A5-11*), which perform as functional transporters only upon interaction with members of the *SLC3* gene family (Verrey *et al.*, 2004). On the contrary, it may also be that CIP1 does not hold any transport activity, but itself is required for regulation of the N(K)CC- and KCC- mediated cotransporter activity. Indeed, CIP1 has been reported to form heteromers with and inhibit transport activity of the NKCC1 protein in human embryonic kidney (HEK) 293 cells (Caron *et al.*, 2000). Moreover, recent data suggest that CIP1 may interact with and increase $^{86}Rb^+$ flux mediated by KCC2 in HEK293 cells (Wenz et al., 2009). However, experiments failed to detect interaction of CIP1 with other CCC members- NKCC2 and KCC1 transporters (Caron *et al.*, 2000), and even more, another group failed to confirm CIP1 interaction with NKCC1 (Parvin *et al.*, 2007). The *Drosophila* homolog of the *SLC12A9* gene is strongly expressed in embryos (particularly in larval brains), as well as in adult bodies (Filippov *et al.*, 2003). A specific deletion of the gene did not affect survival rates in response to different salt concentrations (Filippov *et al.*, 2003). Thus, CIP1 may render a rather subtle regulatory effect on the transport activity mediated by other CCC proteins.

The *SLC12A8* gene encoding the CCC9 protein was associated with psoriasis-susceptibility locus on chromosome 3q21 (Hewett *et al.*, 2002). The original as well as all subsequent papers predicted only eleven TM segments for the

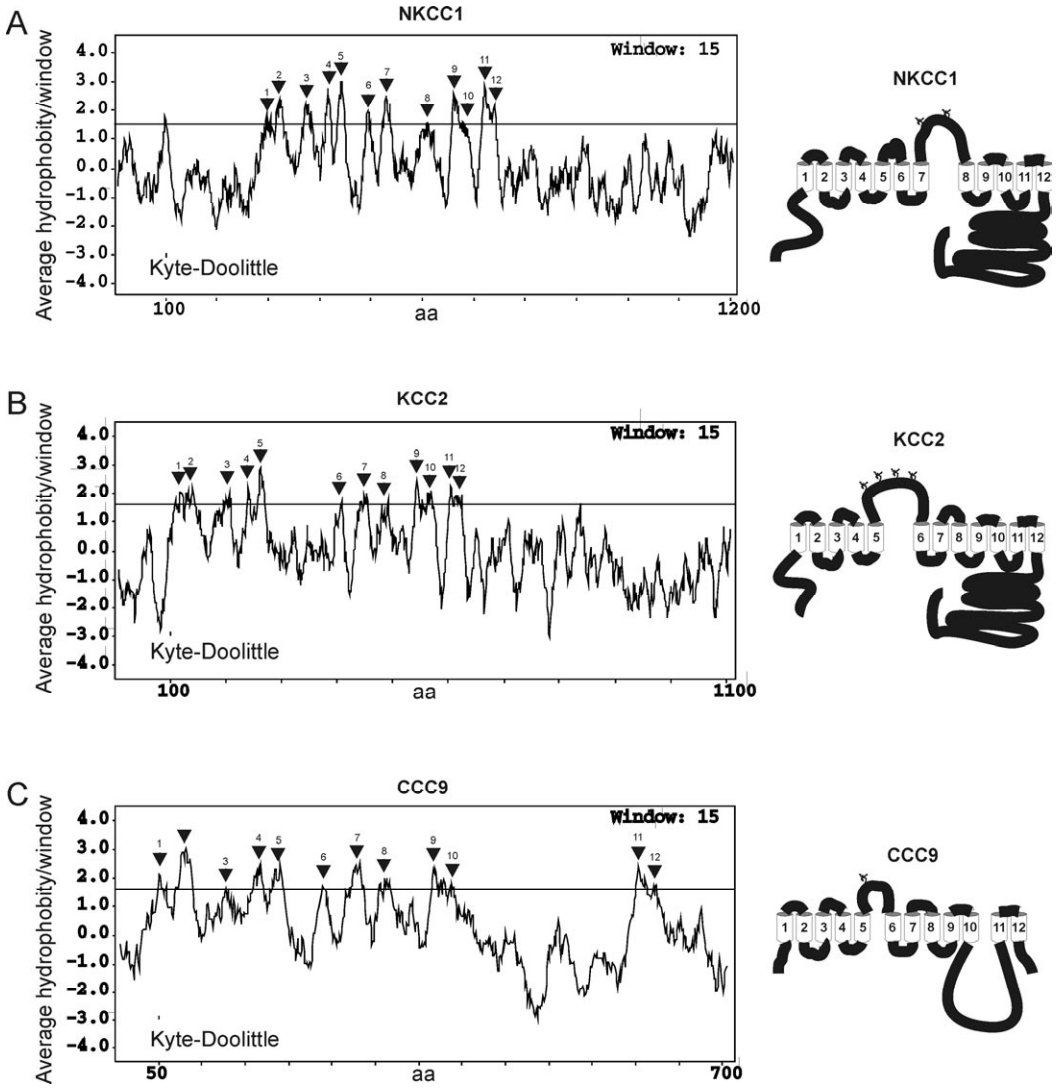

Figure 6. Hydropathy plots for NKCC1, KCC2, and CCC9 proteins. The Kyte-Doolittle hydropathy analysis (on the left) does not rule out for the CCC9 protein (**C**) the same 12-TM structure that has been previously proposed for NKCC1 (**A**) and KCC2 (**B**) members. Schematic model of the NKCC1 (**A**), KCC2 (**B**), and CCC9 (**C**) proteins with 12 TM segments is presented on the right. The window parameter used in the presented Kyte-Doolittle analysis was 15 aa.

CCC9 protein (Fig. 1). However, analysis of the Kyte-Doolittle hydropathy plots does not exclude the common for all CCCs 12-TM structure with small N- and C-terminal domains and a large cytoplasmic loop between the 10^{th} and 11^{th} TM segments (Filippov *et al.*, 2003) (Fig. 6). Similar to CIP1, the CCC9 protein does not mediate Na^+, K^+, or Cl^- transport activity. Examining of its predicted N- and

C- terminal domains suggests that they are much shorter than those in the N(K)CC or KCC proteins. It has previously been shown that deletion or significant shortening of either the N-terminal (Casula *et al.*, 2001; Li *et al.*, 2007) or the C-terminal (Plata *et al.*, 1999; Hekmat-Scafe *et al.*, 2006) domains in the CCC proteins may result in their partial or full inactivation and even in acquisitioning by these truncated proteins the dominant negative properties. Thus, one possibility is that the currently known CCC9 protein sequence is incomplete. Indeed, one of the recent NCBI entries (XM_516709) predicts a significant extension for the *SLC12A8* gene increasing the N-terminal part of the CCC9 protein totally by 227 aa. Another possibility may be that CCC9 uses other substrates for transport. In agreement with this hypothesis, a recent report from Paul Isenring's group has indicated that the CCC9 protein (the original short version) might be involved in polyamine and amino acid transport (Daigle *et al.*, 2009).

An open question is whether CIP1 and CCC9 should belong to the CCC family. So far these proteins have failed to demonstrate any cation-dependent Cl^- transport - a property inherent for other CCC members. Proteins from many other SLC families (Jack *et al.*, 2000; Hediger *et al.*, 2004) also contain a similar 12-TM structure. In particular, a phylogenetic analysis of human Refseq proteins disposes CIP1 and CCC9 outside the N(K)CC/KCC branches (Fig. 2). A relatively low sequence similarity (~20-25%) with other CCC members does not allow prescribing unequivocally CIP1 and CCC9 proteins to the CCC family. One member of the cationic amino acid (CAT) family – CAT-2 protein (Park & Saier, 1996), encoded by a *SLC7A2* gene, reveals the highest homology to the CCC members (Fig. 2). The recent finding that CCC9 uses amino acids and polyamines as a substrate implies that CCC9 may possibly belong to one of the previously described amino acid transporter families. In this connection, it will be interesting to investigate whether CIP1, similar to CCC9, can transport other substrates. It might also be interesting to test whether CCC9, similar to CIP1, is able to regulate the transport activity of other CCC members. In any case, more experimental data are needed to answer these questions and to reveal the biological significance of CIP1 and CCC9 proteins.

1.2.4 CCC diversity: implications

Bioinformatics analysis of the available data on the different CCC splice isoforms has several implications. First, analysis of the multiple N- and C-terminal CCC variants has confirmed the importance of the N- and C-terminal domains in regulating the transport activity of CCC proteins. Truncation of the most C-terminal sequences, which are highly conserved among CCC members, often results in functional inactivation of the cotransporters. The situation with the N-terminal variants is more puzzling. On the one hand, there is a tremendous heterogeneity in the N-termini of different CCC proteins, thus decreasing the chance to find any conserved structural motifs. On the other hand, N-terminal truncations lead to functional inactivation of the cotransporters (Casula *et al.*, 2001; Li *et al.*, 2007), thus indicating that these sequences are important. Analysis of the CCC proteins revealed the presence of many putative phosphorylation sites in the N-terminal parts. It is also possible that

some N-terminal sequences are required for correct processing, transport, and/or incorporation of the cotransporters into the plasma membrane.

Second, simultaneous analysis of all CCC members together with their multiple isoforms may help to understand the role of different structural parts of CCC proteins involved in ion transport. Up to recently, no crystal structure for any of the CCC members has been obtained, and there is no convincing model describing what parts of the cotransporters may be responsible for ion binding (Gamba, 2005). Nevertheless, multiple alignments of CCC protein sequences present in the Ensembl Database (ensembl.org), including all splice variants from different species (totally 241 sequences for the KCC branch, and 216 sequences for the N(K)CC branch), suggest several potential regions for ion binding (Fig. 7A and 7B). Figure 7A depicts the rat KCC2a protein sequence with amino acid positions that are conserved in 87% out of 241 analyzed sequences. The most conserved region occurs to be a part of the second intracellular loop between TM2 and TM3 together with the whole TM3 segment. Interestingly, an analogous analysis performed for the N(K)CC sequences (87% conservation, 216 sequences) identified the most conserved amino acids also in the intracellular loop between TM2 and TM3, but in addition in TM1 and TM6 (Fig. 7B). Although the analyzed sequences belong to species as distinct as *C.elegans* and *H.sapiens*, they still share one common feature – cation-coupled Cl^- transport. This suggests that these regions are important for ion binding/transport across the plasma membrane. One could also speculate that the identified conservation of the KCC proteins in the second intracellular loop and TM3 is important for K^+-driven Cl^- extrusion. On the other hand, TM1 and TM6 regions as well as the second intracellular loop in N(K)CC proteins are central for the Na^+-driven inward Cl^- transport.

Third, the analyzed splicing data will be used in the subsequent chapters to interpret results obtained from the existing CCC knockout (KO) models, especially when a genetic targeting of an alternatively spliced exon has been performed. Disruption of the first exon might result in inactivation of only the isoform containing the targeted exon. Given that different isoforms may display different expression, regulatory, and/or kinetic properties, information on what exon exactly has been targeted is crucial for the interpretation of the KO mouse data, as in the case of the KCC2b KO mice (see below).

Fourth, the existence of multiple CCC variants should also be kept in mind when interpreting published data on spatial and temporal expression of these isoforms. For example, the KCC4 (Karadsheh *et al.*, 2004) and KCC2 (Hubner *et al.*, 2001) antibodies characterized previously would recognize only the KCC4b and KCC2b isoforms, respectively, but not the KCC4a and KCC2a isoforms. Another example is the popular T4 monoclonal antibody derived against the NKCC1 C-terminal part that is highly conserved among N(K)CC proteins (Cutler & Cramb, 2002). Multiple expression and localization studies that have exploited T4 antibody should be regarded with a great concern, because this antibody seems to recognize differentially the C-terminal splice variants of NKCC1 (Zhang *et al.*, 2007).

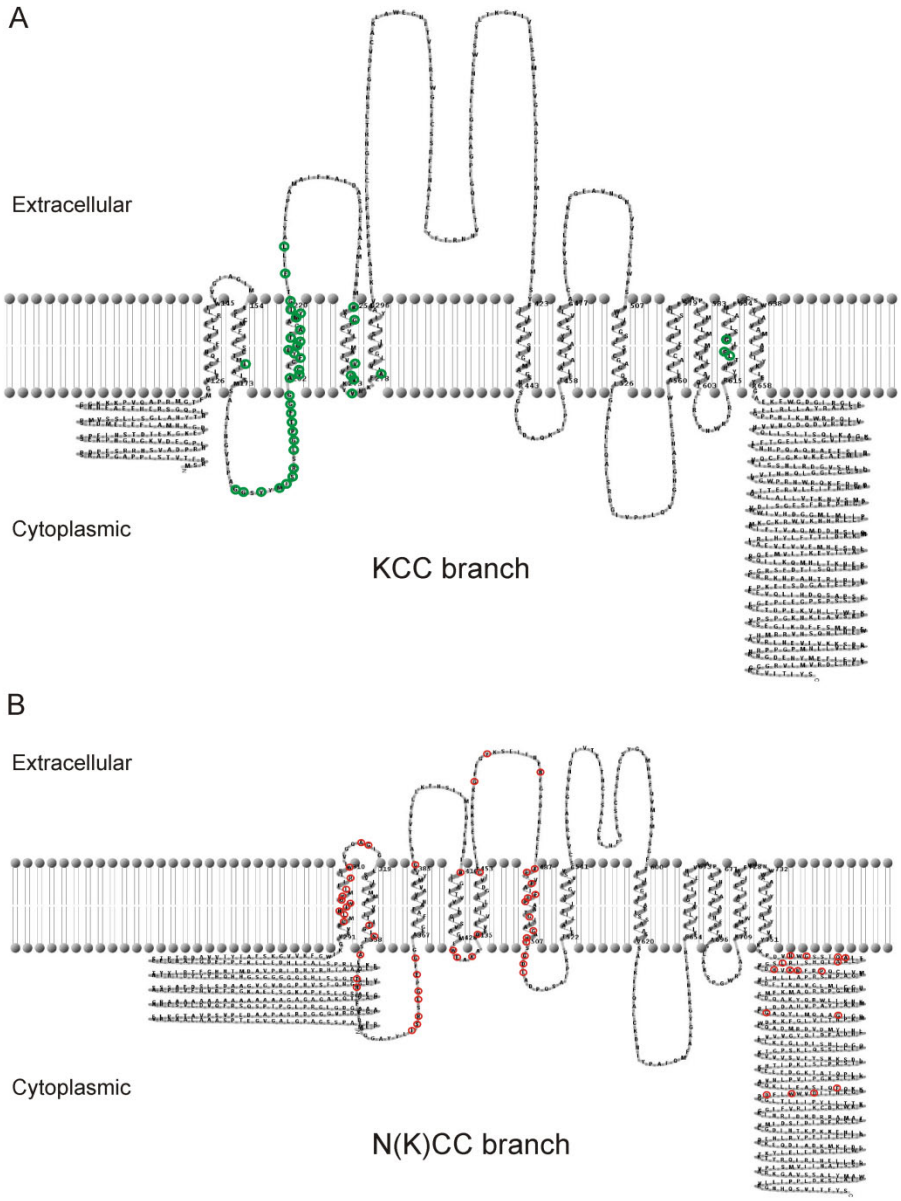

Figure 7. Conservation of the KCC and N(K)CC proteins. (**A**) Schematic drawing of rat KCC2a protein. Amino acid positions marked in green are conserved in at least 87 percent of all KCC sequences available at the moment via Ensembl (totally 241). The most conserved region occurs to be a part of the second intracellular loop between TM2 and TM3 together with the whole TM3 segment. (**B**) Similar analysis performed for the N(K)CC sequences (87% conservation, 216 sequences) identified the most conserved positions (orange) in TM1 and TM6, as well as in the intracellular loop between TM2 and TM3. Rat NKCC1 protein sequence is depicted for illustration.

1.3 SPATIO-TEMPORAL PATTERN OF KCC2 EXPRESSION

1.3.1 Strategies, tools, and precautions

Gene expression can be assessed at three levels: DNA, RNA, and protein. Evaluation of gene expression by analyzing RNA and protein levels is more common and better presented from the methodological point of view. Classical methods to quantify RNA abundance include: Northern blot, ribonuclease protection assay (RPA), reverse transcription followed by polymerase chain reaction (RT-PCR), *in situ* hybridization, *in situ* PCR, and DNA microarray analysis. Protein expression is usually studied by Western blot, co-immunoprecipitation (coIP), and immunohistochemistry (IHC) or immunocytochemistry (ICC). Information on gene activity can be obtained also on the DNA level by analyzing genomic DNA regulatory regions that control transcription: promoters, enhancers, repressors, etc. These regions can be studied by analyzing the methylation status, chromatin modifications, and occupancy by TFs. Among available methods here are: bisulfite sequencing, methyl-sensitive PCR, chromatin immunoprecipitation (ChIP), genome-wide location analysis (ChIP-on-Chip), Whole-Genome Chromatin IP sequencing (Chip-Seq), and analysis of the reporter activity *in vitro* (cell lines) as well as *in vivo* (transgenic mice models).

Many of the aforementioned methods have been utilized so far to study KCC2 expression. However, before reviewing the spatio-temporal expression of the *SLC12A5* gene, encoding the KCC2 protein, the most notorious pitfalls of these methods should be considered. First, RNA-based methods require intact and high quality RNA, thus additional controls ensuring RNA quality are essential; this especially concerns Northern blot analysis. As discussed previously, RNA-based methods may lead to erroneous conclusions if the information about possible splice isoforms is not taken into account. Thus, sequences of PCR primers, as well as of *in situ*, RPA, and DNA microarray probes in all of the KCC2 expression studies performed so far need to be carefully verified. Moreover, possible formation of RNA hairpin structures in regions detected by RT-PCR primers, RPA and *in situ* probes may reduce intensity of the signal and thus affect interpretation of the results. Putative antisense transcripts produced by pseudogenes (Korneev *et al.*, 1999) or by additional promoters (Morris *et al.*, 2008) may distort gene expression data, and thus have to be analyzed as well.

Pitfalls of the protein-based quantification methods are mainly associated with specificity of antibodies. The type of tissue fixation (paraformaldehyde (PFA), methanol, etc) and embedding (cryo, paraffin), various strategies of epitope retrieval (e.g. microwave heating), and different sensitivity to permeabilizing reagents (NP40, Nonidet-P40, Triton-X100, SDS, etc) greatly affect the performance of antibodies (Fritschy, 2008). Inclusion of proper positive and especially negative controls is absolutely necessary to ensure specificity of the expression pattern obtained via usage of a particular antibody. Thus, to analyze gene expression a good strategy is to collect data on several levels (DNA, RNA, and protein), and preferably to use various methods on each of the levels.

1.3.2 Characteristic features of the *SLC12A5* gene expression

A vast amount of information concerning KCC2 expression has accumulated since the first KCC2 paper was published (Payne *et al.*, 1996). To rationalize this data, the following three general rules have been formulated in recent reviews dedicated to KCC2 expression (Ben Ari, 2002; Mercado *et al.*, 2004; Rivera *et al.*, 2005; Gamba, 2005; Blaesse *et al.*, 2009):

1) KCC2 is expressed only in neurons of the CNS (exclusively CNS-specific pattern).
2) As a whole, KCC2 expression is upregulated during embryonic and early postnatal CNS development (developmental upregulation).
3) In adulthood, only a few subpopulations of CNS neurons are devoid of KCC2 (broad expression in adult CNS).

Below an attempt will be taken to ensure whether and how well the original KCC2 expression data conform to these three rules. Another reason to re-review the available literature on the KCC2 spatio-temporal expression is to reveal possible mechanisms mediating the exclusive CNS specificity and the developmental up-regulation of KCC2.

1.3.3 KCC2 expression: CNS neuronal specificity

1.3.3.1 Rodents

First evidence that KCC2 is not expressed in nonneural tissues emerged from the original paper published by John Payne and coauthors (Payne *et al.*, 1996). By using Northern blot analysis with a 783-bp probe corresponding to the 3'-UTR region, KCC2 mRNA was detected in rat brain, but not in seven nonneural tissues (colon, heart, kidney, liver, lung, spleen, stomach). RT-PCR analysis, designed to amplify an exon1-exon4 fragment of KCC2, failed to detect KCC2 mRNA in rat primary astrocytes as well as in C6 and PC12 cell lines, which were derived from rat peripheral nervous system (PNS), thus suggesting even stricter – CNS neuronal pattern of the *SLC12A5* gene expression. At the protein level, the neuronal specificity was later confirmed by Payne's team via successful development of an anti-KCC2 antibody against a 112-aa (amino acids 932-1043) C-terminal fragment of rat KCC2 (Williams *et al.*, 1999). Western blot analysis using this antibody detected KCC2 protein in neural (cortex, hippocampus, cerebellum, brainstem, and spinal cord), but not in nonneural (skeletal muscle, heart, lung, liver, kidney, and testis) lysates. Of note, KCC2 protein was not found in the sciatic nerve, thus excluding KCC2 expression in peripheral glial cells. Astrocytes and various cell lines of neural origin (C6, SH-SY5Y, GT1-7, PC12, NG-108, and N1E-115) also failed to demonstrate KCC2 protein expression. IHC analysis exhibited no colocalization of the KCC2 protein with glial fibrillary acidic protein (GFAP) in rat cerebellum, thus confirming again the exclusively neuronal expression (Williams *et al.*, 1999). In addition, KCC2 protein was not present in glial cells derived from primary chick retinal cultures.

The exclusively CNS-specific pattern of KCC2 mRNA expression throughout adult rat brain was next confirmed in a detailed *in situ* hybridization study performed by Sato's group, who used two 36-bp anti-KCC2 probes complementary to either exon 9 or 24 (Kanaka *et al.*,

2001). Neither of these probes was able to detect KCC2 expression in choroid plexus. Later, Okabe and coauthors used the same *in situ* probes and confirmed once more the CNS-specific pattern of the KCC2 mRNA expression in hippocampus (Okabe *et al.*, 2003). Absence of KCC2 mRNA in the choroid plexus has been confirmed by RT-PCR (Brown *et al.*, 2004).

Another study of KCC2 mRNA and protein expression in developing mouse, starting from embryonic day (E) 10.5 up to postnatal day (P) 15 confirmed the exclusive CNS specificity of the *SLC12A5* gene expression (Stein *et al.*, 2004). No KCC2 transcripts were detected in peripheral ganglia or other parts of PNS with a long (about 500 bp) *in situ* hybridization probe spanning through exons 3 to 7. In addition, KCC2 mRNA was absent in CNS large white matter fiber tracts like corpus callosum and anterior commissure, thus pointing at the neuronal specificity of the expression (Stein *et al.*, 2004). In agreement with this, no KCC2 expression was detected in glia of cochlear nucleus (Vale *et al.*, 2005), in erythroid cells (Crable *et al.*, 2005), and in cultured mouse cortical astrocytes (Molinaro *et al.*, 2008). RT-PCR with primers specific to exons 24 and 25, showed no KCC2 mRNA expression in nonneural tissues (kidney, liver, and pancreas), while detecting strong KCC2 signals in rat cerebellum and hippocampus (Le Rouzic *et al.*, 2006). Moreover, the same study detected KCC2 transcripts in pure neuronal cultures, but not in mixed glial or in astrocytic cultures, thus again confirming the neuron-specific pattern of KCC2 expression.

1.3.3.2 Human

The CNS-specific pattern of human KCC2 expression was first reported by Mount and coworkers (Song *et al.*, 2002). Northern blot analysis with a 3'-UTR probe detected very clear KCC2 mRNA expression in brain, but not in seven nonneural tissues. In agreement with the previous results (Payne *et al.*, 1996), KCC2 transcripts were not detected in several cell lines (NT2, HUVEC, K562, and JEG3). Interestingly, neuronal differentiation of the teratocarcinoma NT2 cell line, which was derived from human testicular cancer, by retinoic acid into NT2-N cells resulted in prominent KCC2 expression as detected by the same 3'-UTR probe (Song *et al.*, 2002).

Absence of KCC2 expression in the undifferentiated NT2 cells has been recently confirmed on the DNA level via a histone mapping of human chromosome 20q13.12 (Akan *et al.*, 2009). Using a nonbiased ChIP-on-Chip method, the authors revealed a strong enrichment in tri-methylated lysine 27 of histone H3 (H3K27me3) throughout the *SLC12A5* gene. The H3K27me3 modification is known to be associated with transcriptionally repressed genomic regions (Cao *et al.*, 2002). Accordingly, neither KCC2 mRNA expression nor *SLC12A5* gene promoter activity was detected in NT2 clone D1 (NT2/D1) (Akan *et al.*, 2009). Interestingly, Akan and coauthors found that the 3'UTR region of the *SLC12A5* gene is situated within a CpG island, which are often associated with promoter regions. In addition, the 3'UTR region of the *SLC12A5* gene was moderately enriched with H3K4me3 and H3K4me2, which in their turn are known to be associated with potentially transcribed regions. The authors suggested the

presence of a new putative promoter in the 3'UTR region that may drive an antisense RNA transcription to ensure the effective silencing of the *SLC12A5* gene in non-neuronal cells (Akan *et al.*, 2009).

As for other human cells, the absence of KCC2 protein and mRNA expression was detected in corneal epithelial cells (Capo-Aponte *et al.*, 2007), in lens epithelial B3 (HLE-B3) cell line, and in human cataractous lens tissues (Misri *et al.*, 2006).

1.3.4 KCC2 expression: exceptions from the CNS neuronal specificity

1.3.4.1 KCC2 expression outside nervous system

The first report suggesting that KCC2 mRNA might also be expressed in nonneural tissues, even though at a very low level, came from Friauf's group (Balakrishnan *et al.*, 2003). RT-PCR analysis with 3'-UTR KCC2 primers detected KCC2 transcripts in kidney, heart, lung, stomach, and liver in P0 and P16 rats. However, KCC2 mRNA expression in neuronal samples, although not quantified, was clearly much stronger.

KCC2 mRNA expression was also detected in A549 human pulmonary cells by microarray (Malard *et al.*, 2007). The authors demonstrated that KCC2 mRNA expression was upregulated upon exposure of the cells to cobalt. The time course of the KCC2 mRNA expression after cobalt application was studied by real time RT-PCR. Unfortunately, both primers utilized in the study belong to exon 26, thus amplification of genomic DNA or presence of antisense transcripts derived from a putative 3'UTR promoter (Akan *et al.*, 2009) cannot be excluded. Two studies also exploited a microarray approach and reported presence of KCC2 mRNA in oocytes (Bermudez *et al.*, 2004;Lee *et al.*, 2008), but these data have not been confirmed by other methods so far.

KCC2 mRNA expression has been detected in a human FHL124 cell line by RT-PCR (Lauf *et al.*, 2008). This cell line resembles freshly obtained human lens epithelial cells (James *et al.*, 2005). Two primer pairs were used to detect KCC2 in this study (exon6-exon10 and 3'UTR-3'UTR), while a negative control confirmed specificity of the PCR reaction. However, the level of KCC2 mRNA expression in these cells remains unclear. Another example of KCC2 expression in a nonneuronal cell line was presented by Kerschbaums' group (Zierler *et al.*, 2008). A KCC2 cDNA fragment was amplified from BV-2 microglial cells with primers spanning exons 7 and 9, and identity of the KCC2 product was confirmed by sequencing. Moreover, negative controls were performed to exclude contamination or amplification from genomic DNA (Zierler *et al.*, 2008).

Additional evidence for possible KCC2 expression outside the nervous system came from an unconventional direction. Two papers, using nonbiased approaches, reported that the KCC2 gene might play an important role in hepatitis C virus (HCV) replication (Ng *et al.*, 2007; Tai *et al.*, 2009). In the first paper, a high throughput screening of a small interfering ribonucleic acid (siRNA) library was performed in HCV infected NS3-NS5B cells (derivative of human hepatoma cell line Huh7) to identify genes important for replication of the virus. NS3-NS5B cells transfected with SMARTpool siRNA (consisting of four individual siRNAs against each of about 4000 human genes) were first analyzed by secreted alkaline

phosphatase (SEAP) reporter assay. The activity of the SEAP reporter is known to be proportional to the replication of HCV RNA; more than 200 siRNAs significantly inhibited HCV replication and were chosen for a further analysis. At the second step, these siRNAs were again transfected into the NS3-NS5B cells, but now real-time PCR was used to quantify RNA copy numbers both of HCV and of a housekeeping gene β-actin. From the more than 200 initial hits only 9 host genes were identified to be involved in HCV replication. Two of the identified genes were *SLC12A4* and *SLC12A5* genes, which encode KCC1 and KCC2 proteins, respectively. *SLC12A4* and *SLC12A5* siRNAs inhibited HCV replication by more than 90%. The ability of the KCC2 siRNA to efficiently inhibit HCV replication was recently confirmed by an independent study (Tai *et al.*, 2009).

1.3.4.2 KCC2 expression in PNS

One initial challenge against the CNS-specificity of KCC2 expression was a report by the group of Eric Delpire (Lu *et al.*, 1999). This study used an anti-KCC2 antibody against almost the same C-terminal fragment (amino acids 931-1034) of the KCC2, as previously produced by John Payne (Williams *et al.*, 1999). Although IHC of rat brain sections with this antibody confirmed the absence of KCC2 protein in glial cells, surprisingly, it detected KCC2 in some large dorsal root ganglia (DRG) neurons, thus arguing against the excusive CNS specificity of KCC2 expression. To support their immunostaining results, the authors used RT-PCR followed by Southern blot analysis and demonstrated a strong KCC2 specific signal in DRG (Lu *et al.*, 1999). Yet, the same group has recently reported that KCC2 mRNA is expressed neither in mouse nor in rat DRG (Geng *et al.*, 2009). This time, cDNA from mouse brain served as a positive control and produced a strong signal as detected by RT-PCR with exon-1 and exon-4 specific primers.

A barely detectable KCC2 mRNA expression in rat DRG was reported by Kaila's group (Rivera *et al.*, 1999), who used the same PCR primer pair (exon1-exon4) as in the paper previously published by Payne and coauthors (Payne *et al.*, 1996). In contrast to these results, Kanaka et al. could not detect KCC2 mRNA expression in rat DRG neurons (Kanaka *et al.*, 2001). One explanation for this discrepancy is the different sensitivity of the methods used: *in situ* hybridization with a 36-bp long oligonucleotide probe (Kanaka *et al.*, 2001) is substantially less sensitive than RT-PCR (Rivera *et al.*, 1999). Alternatively, it is possible that a small contamination by spinal cord tissue may be left in the isolated DRG.

By using Western blot with an anti-KCC2 antibody (Upstate Biotechnology), Coull and coauthors found very strong KCC2 immunoreactivity (KCC2-ir) in the superficial dorsal horn neurons, while KCC2 signal was absent in DRGs (Coull *et al.*, 2003). Upstate Biotechnology produces two anti-KCC2 antibodies: one is directed against the C-terminal part of the KCC2 protein, while the other is directed against the most N-terminal 18-aa peptide of KCC2b; unfortunately, the authors did not specify which antibody they used. To exclude the possibility that the KCC2 protein is shuttled away from DRG cell bodies to central terminals of primary afferents, Coull and coauthors used electron microscopy and demonstrated the absence of KCC2-ir in central

boutons of synaptic glomeruli in laminae I and II (Coull *et al.*, 2003).

Expression of all CCC members in rat DRG was recently analyzed by Möhrlen's group using a semiquantitative RT-PCR (Gilbert *et al.*, 2007). They found a strong expression of CIP1, KCC1, KCC3, KCC4 and NKCC1, much weaker expression of CCC9, KCC2 and NCC, while no expression of NKCC2, which is known to be exclusively kidney specific. KCC2 primers effectively amplified an exon6-exon10 fragment in this experiment. Moreover, IHC with an anti-KCC2 specific antibody (Santa Cruz Biotech, #sc-19420, goat) revealed weak KCC2 protein expression in DRG, supporting the RT-PCR results. The same authors one year later confirmed their results on KCC2 expression in DRG neurons (Funk *et al.*, 2008). These two last reports are in line with the results of Rivera and coauthors (Rivera *et al.*, 1999) suggesting a weak but still detectable KCC2 expression in rodent DRG.

An attempt to measure quantitatively the difference between KCC2 expression in DRG and other neuronal samples was performed by the group of Homayouni (Ledoux *et al.*, 2006), who used a microarray approach to search for genes that are enriched in adult mouse spinal cord compared to DRG. The authors selected totally 112 genes that demonstrated at least 10-fold stronger expression in spinal cord versus DRG samples, and *SLC12A5* was among the most enriched genes (fourth in the list). According to the microarray data, KCC2 expression was 153-fold stronger in spinal cord compared to DRG. These data probably explain the abovementioned contradictory reports on KCC2 expression in DRG: KCC2 mRNA and protein expression in DRG is very low compared to either brain regions or spinal cord, and can be detected only by relatively sensitive methods like microarray analysis and RT-PCR, but not by less sensitive methods like *in situ* hybridization or Western blot.

Another interesting example of putative KCC2 expression outside CNS is the olfactory epithelium. The olfactory system is known to be comprised of the olfactory epithelium, which resides in the PNS, and the olfactory bulb, which resides in the CNS (Barnett, 2004). Transformation of an odor stimulus into a receptor current is mediated by Ca^{2+}-activated Cl^- channels: the resulting outward Cl^- current accounts for about 90% of the transduction current in isolated mouse olfactory receptor neurons (ORNs) (Boccaccio & Menini, 2007). The depolarizing responses of ORNs require an active mechanism to accumulate Cl^- inside the neurons. In an attempt to identify transport proteins mediating the Cl^- accumulation, the group of Steven Kleene exploited RT-PCR to screen for 21 Cl^- transporters (5 *SLC4* anion exchangers, 9 *SLC12* cation-chloride cotransporters, and 7 members of *SLC26* family) in mouse nasal tissue containing the olfactory mucosa (Nickell *et al.*, 2007). RNA transcripts of 20 transporters, including KCC2, were effectively detected, and the identities of the PCR products were confirmed by sequencing. The primers used for the KCC2 amplification span exons 13 to 17, thus eliminating the possibility of genomic DNA detection. KCC2 expression in the nasal mucosa was rather weak compared to that in cerebellum (positive control) (Nickell *et al.*, 2007). These results are in line with a microarray analysis of highly enriched preparations of mature ORNs, in

which the expression of KCC2, KCC3, KCC4, CIP1, PAT1, AE2, and NKCC1 was detected (Sammeta *et al.*, 2007). In contrast to these reports, the group of Thomas Gensch was not able to detect KCC2 transcripts in rat olfactory sensory neurons by amplifying the exon10-exon16 KCC2 fragment (Kaneko *et al.*, 2004).

KCC2 mRNA expression outside the CNS was also reported in rat cochlear by RT-PCR with primers specific to exons 3 and 5 of the *SLC12A5* gene (Yang *et al.*, 2008). A PCR fragment of 248 bp was amplified from the cochlear and brain samples, but not from the negative control sample, in which the reverse transcriptase was omitted during cDNA synthesis. However, the size of the obtained PCR product (248 bp) was smaller than the predicted size (268 bp) of the KCC2-specific PCR product that should have been amplified with the declared primers. Presence of a 250 bp ladder lane on the gel allowed easy detection of this discrepancy. Thus, this report should be treated with caution, as one cannot exclude that a putative KCC2 splice isoform or some unspecific gene fragment was amplified in the experiment.

1.3.4.3 Neuronal versus glial KCC2 expression

The idea that KCC2 is expressed exclusively in neurons but not in glia has also been challenged (Malek *et al.*, 2003). By using an anti-KCC2 antibody (Chemicon, fragment 932-1043 aa), the authors detected KCC2 expression in cell bodies of oligodendrocytes in rat optic nerve. However, the only negative control for the staining was an omission of the primary antibody that showed no unspecific signal.

1.3.5 CNS neuronal specificity of KCC2: conclusions

Even though most of the reviewed data are consistent with an exclusive CNS neuron-specific pattern of KCC2 expression, several reports suggest that some exceptions from this rule may exist in cell lines (Zierler *et al.*, 2008), glia (Malek *et al.*, 2003), peripheral sensory neurons (Lu et al., 1999;Rivera et al., 1999;Nickell et al., 2007;Gilbert et al., 2007;Yang et al., 2008;Funk et al., 2008), and even outside nervous system (Balakrishnan *et al.*, 2003;Bermudez *et al.*, 2004;Malard *et al.*, 2007;Ng *et al.*, 2007;Lee *et al.*, 2008;Tai *et al.*, 2009). While reviewing these sometimes controversial results, it is important to take into account the technical aspects. For example, unusually high amount of total RNA (20 μg) compared to standard protocols (1-5 μg) was used by Balakrishnan and coauthors (Balakrishnan *et al.*, 2003) that might had resulted in a significantly increased sensitivity of the PCR detection. Similarly, the extreme sensitivity may explain the apparent KCC2 signal in DRG observed by Lu and coauthors (Lu *et al.*, 1999), as in this case RT-PCR detection was additionally enhanced by subsequent Southern blot hybridization. In some cases (Yang *et al.*, 2008), it was not clearly stated whether the obtained KCC2 PCR fragments were sequenced, thus the identity of these products remained under a question. This is especially important in this particular case, because the size of the observed PCR product differed from the predicted one (Yang *et al.*, 2008). On the other hand, the absence of proper negative and/or positive controls (especially in protein based methods) does not allow making a final conclusion on the KCC2 expression (Lu *et al.*, 1999). Omitting of

the primary antibody (Malek *et al.*, 2003) is not a good enough control and cannot exclude that some nonspecific signal was still produced by the KCC2 antibody in oligodendrocytes.

siRNA mediated gene knockdown has nowadays become a powerful tool to assay gene function (Kumar & Clarke, 2007), but it is important to remember about the OFF target effects sometimes produced by siRNAs. As an example, two independent papers (Ng *et al.*, 2007; Tai *et al.*, 2009) recently reported a strong inhibition of HCV replication by KCC2 siRNAs in human hepatoma (Huh7)-derived cells. However, these papers did not present any evidence of KCC2 expression in this cell line! Authors of the first paper (Ng *et al.*, 2007) did not study KCC2 expression in their model system at all, while authors of the second paper (Tai *et al.*, 2009) reported to have been unable to detect KCC2, but provided no information about the methods of detection or even whether KCC2 mRNA or protein had been assessed.

Importantly, technical aspects alone definitely may not explain the discrepancy observed by different groups regarding KCC2 expression outside CNS. One report (Song *et al.*, 2002) has demonstrated that KCC2 expression can be detected after the retinoic acid induced neuronal differentiation of NT2 cells, which otherwise show no KCC2 mRNA. This implies, that it might not be the model system per se that once and for all determines whether KCC2 is expressed or not, but it is rather a complex transcriptional regulation that defines whether *SLC12A5* gene is fully silenced or actively transcribed in the particular system.

To conclude, the reviewed literature shows that KCC2 expression is substantially CNS neuron-specific, although weak KCC2 expression in some PNS neurons (in DRG and olfactory epithelium), and in some cell lines cannot be excluded. Unfortunately, lack of experimental details and proper controls in some of the reviewed reports do not allow making a final decision, whether the observed KCC2 expression is genuine or represents a methodological artefact. Possible mechanisms mediating the CNS-specificity of KCC2 expression will be reviewed later in this thesis.

1.3.6 KCC2 expression: developmental upregulation

Developmental upregulation of KCC2 expression is well established by numerous experiments and described in multiple reviews (Ben Ari, 2002;Mercado *et al.*, 2004;Rivera *et al.*, 2005;Gamba, 2005;Blaesse *et al.*, 2009). Different model systems – **human** (Dzhala *et al.*, 2005), **mouse** (Li *et al.*, 2002;Hubner *et al.*, 2001;Balakrishnan *et al.*, 2003;Ludwig *et al.*, 2003;Hubner *et al.*, 2004;Stein *et al.*, 2004;Zhu *et al.*, 2005;Fiumelli *et al.*, 2005;Takayama & Inoue, 2006;Liu *et al.*, 2006;Takayama & Inoue, 2007;Simat *et al.*, 2007;Zhang *et al.*, 2007;Delpy *et al.*, 2008;Sipila *et al.*, 2009;Chambers *et al.*, 2009;Bortone & Polleux, 2009), **rat** (Clayton *et al.*, 1998;Lu *et al.*, 1999;Vu *et al.*, 2000;Gulyas *et al.*, 2001;Shimizu-Okabe *et al.*, 2002;Li *et al.*, 2002;Wang *et al.*, 2002;Mikawa *et al.*, 2002;Ikeda *et al.*, 2003;Balakrishnan *et al.*, 2003;Shibata *et al.*, 2004;Lohrke *et al.*, 2005;Vale *et al.*, 2005;Dzhala *et al.*, 2005;Jean-Xavier *et al.*, 2006;Blaesse *et al.*, 2006), **guinea pig** (Rivera *et al.*, 1999), **gerbil** (Milenkovic *et al.*, 2007), **ferret** (Zhang *et al.*, 2006),

chicken (Liu *et al.*, 2006), **turtle** (Sernagor *et al.*, 2003;Zhang *et al.*, 2007), **cat** (Kanold & Shatz, 2006) - have been used to study the KCC2 developmental upregulation. Among these multiple reports only a few have so far observed certain decrease in KCC2 expression level during development (Wang *et al.*, 2002; Lohrke *et al.*, 2005). All other data show that in any CNS region (except for a few areas where KCC2 is not expressed) KCC2 expression appears at some developmental stage, increases up to a certain level, and then remains at that level throughout adulthood. The moment, when KCC2 expression begins, depends on CNS area. Moreover, kinetics of the KCC2 upregulation even for the same brain area may vary dramatically, when different species are compared.

Several important methodological considerations should be mentioned before reviewing the KCC2 expression literature. First one concerns the sensitivity of different methods used to study the KCC2 developmental upregulation. More sensitive methods can reveal the KCC2 expression earlier, thus resulting in a possible discrepancy between different reports. Another problem is that most of the quantitative and semiquantitative methods (e.g. RT-PCR, RNAse protection assay, Northern blot, Western blot, etc) require normalization by either some reference gene (cyclophilin, tubulin, GAPDH, actin, etc) or by total RNA or protein amounts. Usage of the different normalization methods thus also might lead to some discrepancy between different reports.

Despite enormous amounts of data on the developmental upregulation of KCC2 expression, the signals and mechanisms mediating the upregulation are still elusive. Even more, it is not clear whether these mechanisms are the same in different CNS regions. The subsequent literature analysis will be performed mostly for the individual CNS areas, because most of the reports concentrated only on one or a few CNS parts, but rarely embraced the CNS as a whole. This, hopefully, will give some cues concerning putative signals and mechanisms mediating the KCC2 developmental upregulation in these areas.

1.3.6.1 Whole CNS studies

First evidence that KCC2 mRNA levels steeply increase in the developing CNS was demonstrated by the group of Roderic Smith (Clayton *et al.*, 1998). The authors performed RPA by hybridizing total RNA isolated from E15, P0, P7, P21, and adult rat whole brains with a KCC2 specific probe; the RPA results were normalized to cyclophilin. The RPA probe (168 bp) included the KCC2 cDNA sequence corresponding to nucleotides 119-286 of the coding sequence. First data showing that KCC2 protein levels significantly increase in the developing rat brain came from Eric Delpire's group (Lu *et al.*, 1999). Using Western blot analysis, they detected ~4-fold upregulation of the KCC2 protein expression in rat whole brain between P1 and P28; the results were normalized to total protein content. The same authors confirmed previous results obtained by Clayton *et al*. on the KCC2 mRNA developmental upregulation: Northern blot analysis revealed ~7-fold increase in the KCC2 mRNA levels between P1 and adult whole brain samples. Even though no reference genes were used for normalization, equal amounts of total RNA were loaded. Unfortunately, no information on the

sequence of the KCC2 specific probe was provided.

However, *in situ* hybridization studies have indicated that analysis of the developmental KCC2 upregulation from whole brain samples by RPA or Western blot might have certain limitations. The major problem is the spatially restricted pattern of KCC2 expression in the embryonic and early postnatal rodent brain (Li *et al.*, 2002; Wang *et al.*, 2002). Li and coworkers found, that KCC2 mRNA was strongly expressed only in diencephalon and rhombencephalon, but was almost completely absent in telencephalon in E14.5 mouse brain. Moreover, even at P0, the whole cortical area in rat and mouse brains is mostly devoid of KCC2 mRNA (Li *et al.*, 2002). These results were confirmed by Wang and coauthors using rat brain at E18 and P1 (Wang *et al.*, 2002). Thus, the sampling of the whole brain at P0 mixes the areas with high KCC2 expression and those where KCC2 is absent.

One important conclusion suggested by these first reports (Li *et al.*, 2002; Wang *et al.*, 2002) was that KCC2 expression seems to follow the maturation of individual neurons. First neurons arise in rat CNS around E12-E13 in the spinal cord and medulla. Neurons in thalamus, hypothalamus, and amygdale originate on E13-E16, while in neocortex most principal neurons appear only on E16-E18. Moreover, hippocampal pyramidal neurons originate on E17-E19, while dentate granule cells appear mostly after birth (Wang *et al.*, 2002). Indeed, Li and coauthors found that in rat CNS at E12.5 the KCC2 mRNA was primarily expressed in the ventral part of the spinal cord. Later, on E14.5, the expression was already observed in more rostral levels of pons, in hypothalamus and basal ganglia, as well as in the ventrolateral parts of the developing olfactory bulb (Li *et al.*, 2002). Around P0, the KCC2 mRNA levels were high in thalamus, hypothalamus, amygdala, and olfactory bulb, but they were still very low in neocortex and hippocampus (Li *et al.*, 2002; Wang *et al.*, 2002). In contrast, at P15 KCC2 expression in cortex and hippocampus was very strong and almost indistinguishable from that at P40 (Wang *et al.*, 2002). In agreement with this, the group of Fukuda published results on the developmental KCC2 upregulation in rat cortex (Shimizu-Okabe *et al.*, 2002). The authors observed an absence of KCC2 mRNA expression in neuronal progenitors, thus confirming the idea regarding KCC2 as a marker of neuronal differentiation.

Another interesting observation of Li and coauthors was an apparent developmental spreading of KCC2 expression along the dorsoventral axis. At E12.5 the KCC2 expression was detected primarily in the ventral part of the spinal cord. Later, at E14.5, in the diencephalon, the strong KCC2 expression was restricted to the ventral thalamus and the ventral lateral geniculate complex, whereas only moderate levels were observed in the dorsal part. In addition, it was the ventrolateral part of the developing olfactory bulb that revealed strong KCC2 expression at the same age (Li *et al.*, 2002).

The idea that KCC2 expression parallels neuronal differentiation was confirmed by groups of Christian Hübner and Thomas Jentsch (Stein *et al.*, 2004). The authors exploited Western blot and *in situ* hybridization to study the KCC2 developmental upregulation in mouse CNS. The *in situ* hybridization probe corresponded to the KCC2 sequence

downstream of exon-3, whereas their anti-KCC2 antibody (Hubner *et al.*, 2001) recognized the most N-terminal 15-aa KCC2 sequence (15N-KCC2). Western blot analysis confirmed the previous results obtained by Lu and coauthors (Lu *et al.*, 1999). In agreement with Li *et al.*, *in situ* hybridization showed that the upregulation of KCC2 expression in mice started on average two days earlier than in rats. First KCC2 transcripts were detected in the ventral part of spinal cord and in the immature brainstem already around E10.5. At E15.5, KCC2 expression had spread over the transverse section of the spinal cord and the medulla; the adult level of KCC2 expression in spinal cord was reached at birth. In agreement with Wang *et al*., the KCC2 expression pattern in brain after P15 was similar to that in adulthood. Similar to Li *et al*. 2002, Stein and coauthors also found that KCC2 expression developed in a caudal-to-rostral fashion. *In situ* hybridization data, presented by Stein *et al*., were confirmed the same year by Hübner and coauthors (Hubner *et al.*, 2004).

1.3.6.2 Spinal cord

Only a few reports have specifically studied the developmental upregulation of KCC2 in the spinal cord (Hubner *et al.*, 2001; Jean-Xavier *et al.*, 2006;Delpy *et al.*, 2008;Vinay & Jean-Xavier, 2008). First data came from the group of Thomas Jentsch (Hubner *et al.*, 2001). Using *in situ* hybridization with a probe corresponding to nucleotides 59 to 557 (amino acids 63 to 229), the authors demonstrated a strong KCC2 expression already at E12.5 in the ventral but not in the dorsal part of the mouse spinal cord; at E18.5 it became strong throughout the spinal cord (Hubner *et al.*, 2001). These *in situ* hybridization data have been confirmed on the protein level by the group of Pascal Branchereau (Delpy *et al.*, 2008). Using IHC with a C-terminal KCC2 antibody, the authors analyzed the developmental expression of KCC2 protein in mouse spinal cord from E11.5 until P0. KCC2-ir was detected already at E11.5 in the ventral part of the spinal cord, specifically in the future grey matter, but not in the marginal or dorsal parts. Later, the KCC2-ir expanded dorsally: at E12.5 the whole ventral area demonstrated KCC2 expression, at E13.5 the KCC2 signal expanded into the medial part of the dorsal spinal cord, and at E14.5 the whole transverse section of the spinal cord expressed the KCC2 protein, yet still stronger in the ventral part. The gradient of the KCC2-ir along the dorsoventral axis almost disappeared at E16.6 and was not anymore observed at P0 (Delpy *et al.*, 2008). Interestingly, a quantitative analysis of the mean KCC2-ir in the ventral part of the lumbar spinal cord revealed no statistical difference during the course of development between E11.5 and P0. Thus, once started around E11.5 in the ventral spinal cord, KCC2 expression remained there at the same level.

An attempt to reveal mechanisms regulating the KCC2 upregulation in spinal cord has been undertaken by the group of Laurent Vinay (Jean-Xavier *et al.*, 2006). They used IHC to show that KCC2 expression in the adult lumbar spinal cord significantly decreased after transection of the spinal cord at the thoracic level at P0. The authors suggested that the neonatal spinal cord transection, by removing certain signals from brainstem, perhaps prevents or at least delays the developmental upregulation of KCC2 (Jean-Xavier *et al.*, 2006).

To sum up, KCC2 expression in the mouse spinal cord arises around E11 in the ventral part. Between E11 and E16, the expression expands gradually along the dorsoventral axis, and reaches the adult pattern around birth. KCC2 expression seems to remain at the same level in the ventral part of the spinal cord during the development course. The KCC2 up-regulation is ~2 days delayed in rat compared to mouse spinal cord.

1.3.6.3 Brainstem

The earliest KCC2 mRNA expression in the developing mouse brainstem has been detected already at E9.5 by the group of Andrew Lumsden (Chambers *et al.*, 2009). The authors used a microarray analysis to identify genes expressed preferentially in even rhombomeres and the *SLC12A5* gene, encoding KCC2, was identified among such genes. The microarray data were confirmed by subsequent *in situ* hybridization analysis, which detected specific expression of the KCC2 mRNA in rhombomere 4 at E9.5. Expression of genes encoding transcription factors (Hoxb1, Gata2, bHLHb5, and Lmo1), retinoic acid receptor α (RARα), glutathione peroxidase 3 (Gpx3), aldolase C (AldoC), and glycine receptor alpha1 (Glra1) was also enriched in rhombomere 4 (Chambers *et al.*, 2009). Later on, around E14.5-E15.5 KCC2 expression spreads into medulla (Stein *et al.*, 2004) and to more rostral part of pons (Li *et al.*, 2002).

Information on KCC2 upregulation in the postnatal brainstem is more inconsistent. On the one hand, Stein and coauthors, by using immunoblotting with 15N-KCC2 antibody, demonstrated that KCC2 protein levels continually increased in mouse brainstem throughout E15.5, E18.5, P3 and P7 time points (Stein *et al.*, 2004). On the other hand, Friauf's group, by using RT-PCR and Western blot (with a C-terminal anti-KCC2 antibody (Lu *et al.*, 1999)), showed that KCC2 expression was constant in rat brainstem between P1-3 and P12-16 (Balakrishnan *et al.*, 2003). Certain areas of auditory brainstem, namely the superior olivary complex (SOC), were studied in more details by *in situ* hybridization. Among the four major SOC nuclei, the authors detected a minor KCC2 increase in medial nucleus of trapezoid body (MNTB), but no KCC2 upregulation in lateral superior olive (LSO), medial superior olive (MSO), or superior paraolivary nucleus (SPN) (Balakrishnan *et al.*, 2003). Single-cell RT-PCR confirmed the *in situ* hybridization results: all 20 analyzed LSO neurons (10 from P3 and 10 from P12 mice) expressed KCC2 regardless of age (Balakrishnan *et al.*, 2003). Surprisingly, another group failed to confirm these results (Shibata *et al.*, 2004). Using a very similar single-cell RT-PCR approach, these authors found KCC2 expression in every LSO neuron isolated from P13-16 rats and only in 2 out of 7 LSO neurons from P0-3 rats. They suggested that KCC2 mRNA levels in neonatal LSO neurons were too low to be detected by single-cell PCR. However, Friauf's group gave further support to their results by analyzing KCC2 expression in SOC nuclei by IHC (Lohrke *et al.*, 2005). Using a polyclonal KCC2 antibody from Upstate, Lohrke and coworkers observed downregulation of the KCC2-ir signal between P0 and P8 in LSO, MSO, and SPN nuclei, and no change of KCC2-ir in MNTB. Lack of postnatal KCC2 upregulation in the developing brainstem has been further confirmed by the group of

Nothwang (Blaesse *et al.*, 2006). The authors used their own anti-KCC2 antibody against amino acid residues 1-93 of KCC2 (N93-KCC2). Western blot analysis, performed with this antibody, detected similar protein levels in P2 and P30 rat brainstem samples. The KCC2-ir was found to be strong already in P4 rat brainstem and did not change significantly at P12. In agreement with the abovementioned reports, no KCC2 postnatal upregulation was detected in the cochlear nucleus - another part of the auditory brainstem (Vale *et al.*, 2005). The KCC2 protein levels, analyzed by immunoblotting with a polyclonal anti-KCC2 antibody from Upstate, were indistinguishable between P4 and P40 in cochlear nucleus samples. Similarly, no KCC2 upregulation was revealed in the anteroventral cochlear nucleus of gerbil during the postnatal development (Milenkovic *et al.*, 2007).

In sum, KCC2 expression in rodent brainstem first arises in the forth rhombomere around E9.5, spreads throughout the medulla and pons until E15, and finally reaches its adult levels by birth. The apparently inconsistent results in postnatal brainstem may be explained partially by methodological problems. Single cell PCR approach is unreliable to quantify the gene expression at different time points. Even worse, instead of using quantitative real-time PCR, both groups exploited a conventional nested RT-PCR with two rounds of amplification! Usage of different anti-KCC2 antibodies might also be a reason for the observed controversy, given that several isoforms of KCC2 protein exist (see below). However, in general, the analyzed data confirm the hypothesis that KCC2 expression follows neuronal maturation.

1.3.6.4 Thalamus

Only three reports have described KCC2 upregulation specifically in developing thalamus (Li *et al.*, 2002; Wang *et al.*, 2002; Ikeda *et al.*, 2003). In agreement with the hypothesis that KCC2 follows neuronal differentiation, Li with coworkers detected a strong KCC2 mRNA expression in the ventral part of diencephalon in E14.5 mouse brain (Li *et al.*, 2002). During that time, the strong KCC2 expression was restricted to the ventral thalamus as well as to the ventral lateral geniculate complex, while only moderate levels were observed in the dorsal part. Later, KCC2 expression continued to spread throughout diencephalon until birth. After birth, KCC2 expression was unchanged in most thalamic subareas (Wang *et al.*, 2002; Ikeda *et al.*, 2003), but it slightly decreased in ventral posterior thalamic nucleus (Wang *et al.*, 2002). The last report is one of only two cases known when KCC2 is downregulated during postnatal development.

1.3.6.5 Cerebellum

Developing cerebellum is an important model to study mechanisms of KCC2 upregulation. The time course of KCC2 upregulation is prolonged and varies significantly in different cell types and in different cerebellar subregions. Purkinje cells (PCs) – the principal output cells of cerebellum - are the first cells to differentiate in the cerebellar cortex. PCs are generated in the ventricular zone near the 4th ventricle of the brainstem. Newly generated postmitotic PCs migrate radially to accumulate in one layer called the cerebellar plate. Later, PCs become aligned and form the Purkinje cell layer (PCL) (Sanes *et al.*, 2000). Takayama and

Inoue examined the KCC2 protein expression in developing mouse cerebellum using their own anti-KCC2 antibody raised in guinea pig (Takayama & Inoue, 2006) against amino acid residues 1022-1042 of mouse KCC2. KCC2 expression was detected already at E15 in the PCL of the anterior-dorsal part of the cerebellar hemisphere, but not in the vermis (Takayama & Inoue, 2007). At E17, KCC2-ir was observed in all PCs in hemisphere and vermis. Importantly, ventricular zone, intermediate zone, and external granular layer were devoid of KCC2-ir throughout the course of embryonic development (Takayama & Inoue, 2007). To study mechanisms of KCC2 upregulation in PCs, the authors performed a double immunostaining for KCC2 and synaptophysin. IHC results suggested that induction of KCC2 expression in mouse PCs might be closely related to synapse formation: the more synaptophysin-positive dots were found in close proximity to PCs, the stronger KCC2-ir was observed. Moreover, the KCC2 expression pattern in PCs correlated well with the development of climbing fiber innervations on the PCs (Takayama & Inoue, 2007).

Granular cell (GC) progenitors arise in a separate progenitor zone called rhombic lip and leave it around E13 in mouse (Hatten & Heintz, 1995). They migrate over the top of the developing PCs to form at E15 a secondary zone of neurogenesis, called the external granular layer (EGL). The GCs continue to proliferate in EGL until the third postnatal week, generating more of the GC progeny (Sanes *et al.*, 2000). Differentiation of GCs in EGL starts around birth; following mitosis, immature GCs migrate through the molecular layer, through the PCL, and finally, between P5 and P20, arrive into the internal granular layer (IGL) (Espinosa & Luo, 2008). In mouse, they start to form excitatory synapses with mossy fibers around P3, and inhibitory synapses with Golgi cells (Go) after P7. First KCC2-ir was detected in GCs of IGL only on P5, after formation of first excitatory synapses with mossy fiber terminals (Takayama & Inoue, 2006). Importantly, almost all KCC2 expressing cells had formed synapses with mossy fiber terminals at P5. After P5, KCC2 expression in GCs increased constantly and reached its adult level around P21. Thus, Takayama and Inoue suggested that neuronal differentiation per se was not sufficient to induce KCC2 expression in GCs: in addition, excitatory synapses were required as a trigger.

Stellate and basket cells – cerebellar interneurons – are generated similar to PCs in the ventricular zone (Zhang & Goldman, 1996). They proliferate in mouse cerebellum until P7 and migrate into the molecular layer during second postnatal week (Yamanaka *et al.*, 2004). First GABAergic synapses on the stellate/basket interneurons are formed as soon as the cells complete their migration (Simat *et al.*, 2007). Simat and coauthors also found that KCC2 expression in the stellate/basket cells was barely detectable at P10, became prominent at P15, and reached high levels at P20. Thus, similar to PCs and GCs, KCC2 expression in the stellate/basket interneurons arose only in the differentiated cells.

The time course of the KCC2 upregulation in developing rat cerebellum was similar to that in mouse (Li *et al.*, 2002; Mikawa *et al.*, 2002; Blaesse *et al.*, 2006). Premature cells in EGL did not express KCC2 throughout neurogenesis,

while PCs revealed a strong KCC2-ir already at P1 (Mikawa *et al.*, 2002; Li *et al.*, 2002). At P7, KCC2 expression was strong in PCL, but very weak in IGL, which only starts to form at that time. On P14, both the PCL and IGL revealed a strong KCC2-ir; KCC2 expression reached its adult levels in rat cerebellum at P21. In adult cerebellum, KCC2 mRNA was expressed in most neurons, including PCs, GCs, stellate cells, and basket cells (Mikawa *et al.*, 2002).

In sum, analysis of KCC2 expression in the developing cerebellum leads to several conclusions: (1) KCC2 mRNA expression is induced at the same time when neurons arrive at their final destinations (Mikawa *et al.*, 2002), (2) formation of excitatory synapses is required to induce KCC2 expression in a target cell, while the neuronal differentiation per se, although necessary, is not sufficient (Takayama & Inoue, 2006;Takayama & Inoue, 2007), and (3) GABAergic synaptogenesis plays an important role in the maturation of cerebellar interneurons and, as a consequence, in the KCC2 upregulation (Simat *et al.*, 2007). Li and coworkers originally found that not all TUJ1-positive cells (marker of neuronal differentiation) do obligatory express KCC2 (Li *et al.*, 2002). Thus, these new three conclusions don't contradict but rather complement the hypothesis that KCC2 expression follows neuronal maturation.

1.3.6.6 Hippocampus

The first description of the developmental upregulation of KCC2 expression in hippocampus was obtained by Kai Kaila's group (Rivera *et al.*, 1999). Northern blot hybridization with a 635-bp long probe, corresponding to the most 5'-part of KCC2 cDNA, revealed that KCC2 expression in rat hippocampus was barely detectable at P0, rather weak at P5, but very prominent already at P9. Finally, KCC2 expression reached its adult levels around P15. Rivera and coworkers confirmed these results by *in situ* hybridization analysis with a probe corresponding to nucleotides 1-835 of the rat KCC2 cDNA. KCC2 mRNA was detected in hippocampus neither at E20 nor at P0, but was prominent at P5, and very strong at P16 (Rivera *et al.*, 1999). Wang and coworkers, using their own *in situ* probes, obtained very similar results: only very weak KCC2 expression was detected in rat hippocampus at P1, while at P15 it became very strong and comparable to that at P40 (Wang *et al.*, 2002). Additional *in situ* hybridization data came from Friauf's group: at P0 all rat hippocampal areas were devoid of KCC2 signal, while at P16 the labelling was clear in all areas (Balakrishnan *et al.*, 2003). In contrast, Li and coworkers, by using *in situ* hybridization, detected weak but clear KCC2 expression in mouse hippocampus at P0 (Li *et al.*, 2002). This discrepancy may be explained by a slightly delayed development of rats compared to mice.

Additional data on the developmental expression of KCC2 at the protein level in mouse hippocampus came from the group of Christian Hübner (Stein *et al.*, 2004). Immunoblotting revealed no KCC2-specific signals in E15.5 and E18.5 hippocampi, rather weak signal at P3, prominent at P7, and very strong at P15. Even though the KCC2 protein expression at E18.5 was not detected by immunoblotting, at the same time a very weak KCC2 mRNA signal was revealed by *in situ* hybridization (Stein *et al.*, 2004).

A steep increase in the KCC2 protein levels was further confirmed by the group of Darwin Berg, who found that Western blot signals from mouse hippocampal samples were about 4-fold stronger at P9 compared to P6 (Liu *et al.*, 2006). Interestingly, there was no KCC2 upregulation detected between P6 and P9 in KO mice deficient for the α7 subunit of nicotinic acetylcholine receptor. Thus, the authors suggested that nicotinic signalling might play an important role for the developmental increase of KCC2 in hippocampus (Liu *et al.*, 2006).

Recently Sipilä and coworkers, using Western blot analysis with a C-terminal anti-KCC2 antibody (Ludwig *et al.*, 2003), have demonstrated that KCC2 protein expression in mouse hippocampus was up-regulated slightly between P3/4 and P6/7, but steeply (~3-fold) between P6/7 and P20 (Sipila *et al.*, 2009). The authors asked whether the developmental up-regulation of KCC2 depends on the NKCC1-mediated depolarizing actions of γ-aminobutyric acid (GABA). For this they analyzed the developmental course of the KCC2 upregulation in mice deficient for the NKCC1 protein. The authors found that KCC2 expression did not differ significantly in KO compared to wild type mice (Sipila *et al.*, 2009). Similar results were reported by another group (Pfeffer *et al.*, 2009), using a 15N-KCC2 antibody.

Developmental upregulation of KCC2 expression in CA1 and CA3 hippocampal areas has also been studied by Delpire and coauthors, who exploited Western blot with their own anti-KCC2 antibody (Lu *et al.*, 1999), and found that KCC2 expression appeared in CA1 area earlier than in CA3 (Zhu *et al.*, 2008).

The above mentioned *in situ* hybridization and immunoblotting data have been confirmed by two IHC studies (Gulyas *et al.*, 2001; Blaesse *et al.*, 2006). Blaesse and coworkers, using an N93-KCC2 antibody (see above), observed almost no KCC2 signal in P4, but strong expression in P12 rat hippocampus. In addition, detailed IHC analysis of the developmental KCC2 expression in rat hippocampus was performed by Gulyas and coauthors. Only very faint and diffuse KCC2-ir was detected in the hippocampus at P0; later, KCC2 expression gradually increased and reached adult levels at P12. Interestingly, similar to the situation in cerebellum, the authors found that the KCC2 upregulation possibly takes place in an input-specific manner: KCC2 expression in DG neurons correlated with the level of innervation of these neurons by entorhinal cortical axons. The fact that KCC2 expression reached its adult levels at P12 was in agreement with the view that the layer specific inputs formed by the perforant path and the commissural afferents are established by P12 (Gulyas *et al.*, 2001). Another interesting finding mentioned by Gulyas and coauthors was a very strong KCC2 expression in parvalbumin (PV)-containing inter-neurons. These cells receive in average about 8 times more excitatory input compared to other interneurons (Gulyas *et al.*, 1999). Whether the significantly increased KCC2 expression in the PV-containing interneurons was a result of this strong excitatory input remains to be determined.

One hypothesis to explain the developmental upregulation of KCC2 expression was suggested by the group of Fernando Aguado (Aguado *et al.*, 2003). They generated mice overexpressing brain-derived neurotrophic factor (BDNF) and demonstrated that KCC2 mRNA

expression was ~4-fold higher in the E18 transgenic embryos compared to their wild type littermates; semiquantitative RT-PCR was performed with primers corresponding to nucleotides 4-479 of the mouse KCC2 coding sequence, and normalized to GAPDH. *In situ* hybridization with a probe obtained with the same primers revealed almost no KCC2 mRNA in hippocampus of wild type embryos at E18, but detected high levels of KCC2 expression in the littermates overexpressing BDNF, thus confirming results of the RT-PCR (Aguado *et al.*, 2003). Few years later, the same group generated mice deficient for the tropomyosin receptor kinase B (TrkB) – the receptor kinase for BDNF (Carmona *et al.*, 2006). KCC2 expression was assessed in these $TrkB^{-/-}$ mice by Northern blot and by *in situ* hybridization with the KCC2 probe described previously (Aguado *et al.*, 2003). Both methods revealed a moderate reduction of the KCC2 mRNA levels in hippocampal and cortical areas of the P10 $TrkB^{-/-}$ mice compared to wild type littermates (Carmona *et al.*, 2006). The consistent results obtained from the BDNF-overexpressing and TrkB-deficient mice suggest an importance of the TrkB-signalling for the developmental up-regulation of KCC2, although indirect effects of the above manipulations on the TrkB pathway cannot be excluded.

To sum up, KCC2 expression in rodent hippocampus develops mainly during first two postnatal weeks. The mechanisms responsible for the developmental KCC2 upregulation in hippocampus remain unclear. GABA-mediated depolarization seems dispensable for the developmental upregulation of KCC2 (Pfeffer *et al.*, 2009; Sipila *et al.*, 2009). However, glutamatergic activity and intrinsic excitability of CA3 pyramidal neurons in the $NKCC^{-/-}$ animals are enhanced compared to the wild type mice via unknown compensatory mechanism, which may be sufficient to keep KCC2 expression at the level found in wild type mice. Similarly, Gulyas and coworkers observed a correlation between the KCC2 expression level and the excitatory input obtained by neurons, thus also suggesting the importance of neuronal activity for the KCC2 expression during development (Gulyas *et al.*, 2001). The results presented by Aguado *et al*. and Carmona *et al*. may suggest a role for BDNF in regulating the KCC2 induction in the developing hippocampus. However, BDNF-overexpressing mice show increased spontaneous activity, correlated network activity, synaptogenesis, and expression of GABA-synthesizing enzymes glutamic acid decarboxylases 65 and 67 (GAD65/67) (Aguado *et al.*, 2003). Similar problems persist in $TrkB^{-/-}$ mice, as spontaneous activity, number of GABAergic synapses, expression on GAD65/67 enzymes, glutamate and GABA release, as well as expression of several AMPA and $GABA_A$ receptor subunits were also affected in these mice (Carmona *et al.*, 2003;Carmona *et al.*, 2006). Thus, it is difficult to conclude whether the observed changes in KCC2 expression is a direct result of the impaired BDNF signalling or an indirect consequence of other BDNF-mediated changes occurring in brains of these mice.

1.3.6.7 Cortex

The first demonstration of the developmental KCC2 upregulation in cortex was published by the group of Roderic Smith (Clayton *et al.*, 1998). Using *in situ* hybridization, this group

analyzed KCC2 mRNA abundance in E18, P0, P7, P21, and adult rat cortex. No KCC2 mRNA expression was detected at E18, very low signal was seen at P0, but it was significantly increased at P7. High levels of the KCC2 expression were observed at P21 and in adult samples (Clayton *et al.*, 1998). Analogous *in situ* hybridization results were obtained by groups of Fukuda (Shimizu-Okabe *et al.*, 2002) and Sato (Wang *et al.*, 2002). Shimizu-Okabe and coauthors analyzed KCC2 expression in P0, P4, P10, and P28 rat cortex: it was very low at P0-P4, and became significant only after P10. No KCC2 mRNA expression was detected in the ventricular zone at any developmental stage (Shimizu-Okabe *et al.*, 2002). In their turn, Wang and coauthors could not detect the KCC2 expression at P1, whereas it was dramatically increased at P15, reaching its adult levels at that time (Wang *et al.*, 2002). Ikeda and coworkers also used *in situ* hybridization to compare the developmental course (P0, P4, P7, P14, P28) of the KCC2 expression in rat visual cortex to that in dorsal lateral geniculate nucleus (Ikeda *et al.*, 2003). They found that KCC2 mRNA level in visual cortex was very low at P0, but gradually upregulated at P28. In contrast, no change in the KCC2 mRNA level was detected in the geniculate nucleus throughout postnatal development.

Western blot analysis of the KCC2 expression in developing cortex has been presented so far only by two reports (Stein *et al.*, 2004; Dzhala *et al.*, 2005). Stein and coworkers could not detect any KCC2 expression in mouse brain at E15.5, found it very weak at E18.5, prominent at P3, getting stronger at P7, and very strong at P15. Importantly, the KCC2 antibody used in this experiment recognizes the most N-terminal 15 amino acid sequence of the KCC2 protein. Dzhala and coauthors have analyzed developmental upregulation of KCC2 in rat and human cortical samples. In rat, KCC2 expression was first detected at P11 and then steeply increased until adulthood. In human cortex, the KCC2 protein levels were barely detectable during the entire fetal period. In addition, KCC2 expression was about 2-25% of adult levels between gestational weeks 31 and 41, and then increased during the first year after birth (Dzhala *et al.*, 2005).

Kanold and Shatz used cat visual cortex as a model to test the hypothesis that KCC2 expression is regulated by neuronal activity (Kanold & Shatz, 2006). Using *in situ* hybridization and quantitative RT-PCR, the authors showed that KCC2 expression in the cat visual cortex increased gradually from P0 until P35. Intriguingly, the developmental upregulation of KCC2 expression was distorted in subplate-ablated cats. Subplate neurons are known to be innervated by axons from the lateral geniculate nucleus and are among first neurons innervating the cortical layer 4. Ablating of the subplate neurons leads to the decreased synaptic input to the layer 4. In subplate-ablated cats, KCC2 expression substantially decreased in the layer 4, but not in other cortical layers. At P5, the KCC2 mRNA level in the cortical layer 4 was about 70% lower in the subplate-ablated compared to the unmanipulated animals; moreover, a significant (~50%) difference still persisted in cats at P50 (Kanold & Shatz, 2006). Another important result of this paper was that the developmental upregulation of KCC2 in layer 4 was prevented by chronic blockade of glutamatergic transmission. Subplate neurons are known to provide largely

glutamatergic, excitatory inputs to cortex (Finney *et al.*, 1998), thus the developmental upregulation of KCC2 in the cortical layer 4 requires the intact glutamatergic transmission mediated via subplate neurons.

Interesting results on the mechanism of KCC2 expression during cortical development have been recently presented by the group of Gord Fishel (Batista-Brito *et al.*, 2008). They generated transgenic mice expressing enhanced GFP (EGFP) under the Dlx5/6 promoter, thus specifically marking cortical interneuronal precursors. The authors dissected the cortical areas at E15.5, isolated the EGFP-positive interneurons by FACS, and analyzed a genome-wide gene expression profile of the EGFP+ neurons by microarray analysis. Surprisingly, *SLC12A5* gene, encoding KCC2 protein, was found among other genes (including *GAD67*, *VGAT*, *GABARγ2*), whose expression was highly enriched in the cortical interneuron precursor populations at E15.5. The microarray results were confirmed by *in situ* hybridization with a probe that contained mostly the 3'UTR part of the KCC2 (Batista-Brito *et al.*, 2008). These results suggest that cortical interneurons may start to express KCC2 already during their migration, before being fully integrated into a local synaptic network.

In sum, KCC2 upregulation in rodents reaches its maximum levels by the 3rd postnatal week. KCC2 expression is significantly slower in cat and human cortical samples, where the adult levels are reached only at ~P40 and ~1 year postnatally, respectively. Two reports (Ikeda *et al.*, 2003; Kanold & Shatz, 2006) are in agreement with the hypothesis that the developmental expression of KCC2 (at least in cortex) might be increased by neuronal activity. Ikeda and coworkers found that KCC2 expression in the dorsal lateral geniculate nucleus and in the visual cortex correlates with establishing of final axonal projections into these areas. In their turn, Kanold and Shatz revealed that KCC2 upregulation in the cortical layer 4 requires an intact glutamatergic transmission mediated via subplate neurons. In cortical interneurons, KCC2 expression appears to be induced even before neuronal migration is completed (Batista-Brito *et al.*, 2008). Using *in situ* hybridization and microarray analysis, the authors detected prominent KCC2 expression in the migrating cortical interneuronal precursors in the developing cortex already at E15.5. Of note, most previous reports had failed to appreciate KCC2 expression in the cortex before E18.5. Detection of the KCC2 mRNA by *in situ* hybridization may be explained by a high sensitivity of the probe. Another possible explanation is that the 3'UTR probe recognized certain KCC2 splice isoform, but not the full length KCC2 mRNA. This possibility is suggested by the report of Akan and coauthors, who predicted one new KCC2 promoter in the 3'UTR part of the *SLC12A5* gene (Akan *et al.*, 2009). The early expression of KCC2 in migrating interneuronal precursors has been confirmed by Dante Bortone and Franc Polleux, who revealed a weak KCC2-ir at E16.5 in interneuronal precursors, but not in pyramidal neurons of the cortical plate (Bortone & Polleux, 2009). The authors have also determined that even though KCC2 expression in the interneuronal precursors was heterogeneous, it was still much stronger than in pyramidal neuronal precursors. Moreover, KCC2 expression in average appeared in

interneurons about 1 week earlier than in pyramidal neurons. Although the migrating interneurons increased KCC2 expression steeply only upon reaching the cortical area, they did express KCC2 already during their migration from the ganglionic eminence to the cortical area (Bortone & Polleux, 2009).

1.3.6.8 Retina

KCC2 mRNA expression in mouse retina can be detected as early as E14.5 and its levels increase slowly by P0 (Li *et al.*, 2002). Immunoblotting showed that KCC2 expression in rat retina continued to increase after birth: it constituted ~25% of adult level at P3, ~75% at P7, and finally reached the adult level at P14 (Vu *et al.*, 2000). Moreover, IHC analysis detected KCC2 protein first in inner retina around P1, and in outer retina only around P10. KCC2-ir was clearly visible in both inner and outer retina at P14. KCC2 immunoreactivity became more punctuate after P14, but the total expression level did not change much between P14 and P32 (Vu *et al.*, 2000). Whether the delayed KCC2 expression was a reason or a consequence of the delayed synaptogenesis in the outer retina is an open question.

Mechanisms of KCC2 upregulation have also been studied in the inner plexiform layer (IPL) of turtle retina (Sernagor *et al.*, 2003). The authors reported that the fraction of the KCC2-positive IPL constituted ~60% one week before hatching, ~95% around time of hatching, and the same ~95% by 3rd week post hatching. Interestingly, the KCC2-ir fraction was found to be ~20% smaller in the IPL of turtles kept for three weeks post hatching in dark-rearing compared to normal conditions, suggesting a role of neuronal activity for the developmental upregulation of KCC2. In agreement with the proposed role of KCC2 in modulation of GABA responses, GABA retained a substantial excitatory component after 4 weeks of dark-rearing, while in normal conditions the hyperpolarizing GABA responses were observed during the first week post hatching (Sernagor *et al.*, 2003). To address whether GABA-signalling was important for the developmental upregulation of KCC2 in turtle retina, the same group performed a series of experiments, in which $GABA_A$ receptors were chronically blocked *in vivo* by antagonist bicuculline (Leitch *et al.*, 2005). The authors detected a down-regulation of the KCC2-ir inside the IPL after 28 days of the chronic blockade. However, the effect of blockade was rather modest: the intensity of the KCC2-ir decreased only by 15%, and the percentage occupancy of KCC2 within IPL decreased only by 12% (Leitch *et al.*, 2005).

Developmental upregulation of KCC2 in ferret retina was studied by the group of Vardi (Zhang *et al.*, 2006). Immunoblotting with a C-terminal anti-KCC2 antibody demonstrated a low KCC2 protein expression before P28 (less than 10% of adult level) and a very steep upregulation after P28. Totally, the KCC2 protein level increased three orders of magnitude from P10 to adult (Zhang *et al.*, 2006). Interestingly, a similar upregulation course was observed for synaptophysin: KCC2/synaptophysin ratio did not change significantly during the whole developmental period analyzed. IHC confirmed that overall KCC2 expression was upregulated in retina with age, but also revealed that the time course was different for different cell types. A very weak KCC2-ir appeared in the IPL at P3, and

became prominent at P10, when ganglion cell processes start to stratify in the IPL. At P14, the KCC2-ir divided into two strata corresponding to future ON and OFF sublaminae, and at P18 the expression reached a level corresponding to 40% of the adult one (Zhang *et al.*, 2006). Like in the turtle retina, KCC2-ir in the ferret outer retina was significantly delayed compared to the inner retina. Weak KCC2 signal in the outer plexiform layer (OPL) was detected only around P10, and then gradually increased until adulthood. KCC2 expression was more delayed in the bipolar cells. These cells are well-formed already at P28, however no KCC2-ir could be detected before P37 (Zhang *et al.*, 2006).

The same group has also presented a very detailed analysis of KCC2 expression in the developing mouse retina (Zhang *et al.*, 2007). Western blot analysis could not detect KCC2 expression in mouse retina before P8. After P8, the KCC2 levels steeply upregulated peaking at P24 and constituting ~25% and ~80% of the peak level at P12 and P14, respectively. IHC analysis detected KCC2-ir already at P0 in the IPL. At P10, the KCC2 staining divided into two strata corresponding to the future ON and OFF sublaminae (Zhang *et al.*, 2007). KCC2 expression in the outer retina appeared around P7, when OPL is formed, and thereafter the expression continued to increase up to P20. Based on this analysis, Zhang and coworkers suggested that in general KCC2 expression correlated with a differentiation of diverse cell types in retina.

In sum, KCC2 upregulation in retina has been revealed in several model organisms. One common property for all these systems is a very steep course of the KCC2 upregulation. Another common feature is the pronounced delay of the moment when KCC2 expression starts between the inner and outer retina. The fact that the dark-rearing is able to retard KCC2 expression in the IPL points at some light-/activity- dependent mechanism regulating KCC2 expression during retinal development. This activity-dependent mechanism may be partially controlled by $GABA_A$ receptors, as the chronic blockade of these receptors by bicuculline decreases KCC2 expression in the turtle IPL. The relatively modest effect of bicuculline indicates that there are additional mechanisms controlling the KCC2 developmental upregulation in retina.

1.3.6.9 Dissociated neuronal cultures

The dissociated neuronal culture is a popular model to study the developmental shift of GABA responses from depolarizing to hyperpolarizing (D/H shift) that occurs during neuronal maturation. Somewhat surprisingly, the developmental expression of KCC2 – the main proposed molecule responsible for the shift – in the dissociated cultures has been studied only by a few reports. These reports have suggested interesting but contradictory hypotheses on the mechanisms governing the KCC2 upregulation (see below).

A careful characterization of the KCC2 developmental upregulation in mouse hippocampal organotypic and dissociated cultures was performed by Claudio Rivera's group (Ludwig *et al.*, 2003). The authors generated a new C-terminal anti-KCC2 antibody and used it for ICC and for Western blot analysis. Immunoblotting revealed a gradual increase (~5-fold) of KCC2 protein level in mouse hippocampal dissociated cultures

between div4 (day *in vitro*) and div15; KCC2 signals were normalized to β-tubulin. More prominent KCC2 up-regulation (~8-fold) was detected by the immunoblotting in organotypic cultures cultivated from div3 to div14 (Ludwig *et al.*, 2003). ICC showed very similar results: KCC2-ir was very weak in div3 neurons and gradually increased until div15, constituting about 20% at div6, 40% at div8, and 70% at div10 of its maximal level at div15. Additional experiments were performed to clarify the role of neuronal activity and synaptic transmission in the developmental upregulation of KCC2. Surprisingly, a chronic (from div2 to div15) blockade of action potentials, $GABA_A$ receptors, and ionotropic glutamate receptors separately or in different combinations did not influence the KCC2 protein expression. Similar results were obtained for the organotypic cultures. Thus, the authors suggested that other mechanisms (e.g. cell-to-cell or cell-to-matrix interactions) may be necessary for the developmental increase of KCC2 (Ludwig *et al.*, 2003).

Developmental upregulation of the KCC2 protein expression has been observed in dissociated mouse cortical cultures by ICC (Zhu *et al.*, 2005) and in mouse hippocampal cultures by Western blot (Fiumelli *et al.*, 2005). Immuno-blotting, which was performed with the KCC2 antibody from Upstate and normalized to γ-tubulin, detected a 3-fold upregulation of KCC2 between div6 and div15 cultures. Although three bands were seen on the blot - two minor bands of ~140 kDa (corresponding to KCC2 monomer) and one major band of ~240 kDa (putative KCC2 dimer), Fiumelli and coworkers quantified only the KCC2 monomers. Three similar bands were detected by Western blot from lysates of HEK293 cells transfected with a KCC2 expression but not an empty vector, thus proving the specificity of the KCC2 antibody.

An intriguing yet contradictory hypothesis has been suggested by the group of Mu-ming Poo that GABA signalling itself activates KCC2 expression and thus induces D/H shift in GABA responses during neuronal maturation (Ganguly *et al.*, 2001). To test their hypothesis, the authors chronically blocked $GABA_A$ receptors in rat hippocampal dissociated cultures starting at div3 and demonstrated that KCC2 mRNA levels were significantly reduced at div9 (by ~25%) and at div12-15 (by ~70%) in the treated compared to the untreated cultures. It is important to note, that the chronic blockade of $GABA_A$ receptors was unable to completely cease the developmental upregulation of KCC2: KCC2 mRNA levels were still ~4-fold higher in both div9 and div12-15 treated cultures compared to div3 treated cultures (Ganguly *et al.*, 2001). A similar 3-5 fold upregulation of the KCC2 mRNA level was previously described in control untreated dissociated hippocampal cultures (Ludwig *et al.*, 2003; Fiumelli *et al.*, 2005). Of note, in the absence of $GABA_A$ receptor inhibitors, Ganguly and coauthors detected a much more prominent (about 15-fold) increase of the KCC2 mRNA expression between the div3 and div15 cultures. Such a strong KCC2 mRNA upregulation has been reported so far only for hippocampal neurons *in vivo* (Rivera *et al.*, 1999), but neither for dissociated nor for organotypic hippocampal cultures in any other study.

Ganguly and coauthors have not presented data showing that KCC2 mRNA

expression can be directly activated by GABA (Ganguly *et al.*, 2001). Instead, they elevated neuronal, including GABAergic activity, by chronic depolarization of the cultures with 10 mM KCl. This treatment resulted in ~2-fold upregulation of the KCC2 mRNA expression in div9 mouse hippocampal cultures. It is interesting to note, that a similar KCl treatment was unable to change the KCC2 protein expression level in rat hippocampal dissociated cultures as reported by the group of Melanie Woodin (Balena *et al.*, 2008). The authors cultured hippocampal neurons for two weeks in either 2.6 mM or 18.7 mM extracellular KCl, but found no difference in KCC2 protein levels upon the treatment.

To sum up, the available literature has not revealed consensus mechanisms regulating KCC2 expression in dissociated neuronal cultures. On the one hand, a blockade of the GABAergic signalling decreases KCC2 expression in dissociated hippocampal cultures (Ganguly *et al.*, 2001). On the other hand, the KCC2 inhibition observed by Ganguly *et al*. is partial and has not been confirmed by the subsequent study performed in a similar model system (Ludwig *et al.*, 2003). One possible explanation for the controversy is that Ganguly *et al*. used neuronal cultures ~10 times more dense than those used by Ludwig *et al*. It has previously been shown that a higher density of astrocytes in dissociated cultures results in a significant acceleration of D/H shift in cultured spinal cord neurons (Li *et al.*, 1998). Given that ionotropic glutamatergic signalling has not demonstrated any effect on the KCC2 developmental upregulation in dissociated cultures (Ganguly *et al.*, 2001;Ludwig *et al.*, 2003), Ludwig and coauthors suggested a new idea that cell-to-cell or cell-to-matrix interactions may be responsible for the developmental increase of KCC2 (Ludwig *et al.*, 2003). In addition, one common conclusion has been made by both groups: experiments with tetrodotoxin (TTX) exclude the possibility that the KCC2 developmental increase is potentiated by action potentials (Ganguly *et al.*, 2001; Ludwig *et al.*, 2003).

1.3.7 Developmental upregulation of the KCC2 expression: conclusions

The available data clearly demonstrate that KCC2 expression inevitably increases during CNS development. Two reports have observed downregulation of the KCC2 mRNA level during normal development, but only in certain brain regions (Wang *et al.*, 2002; Lohrke *et al.*, 2005). Of note, the time of induction of KCC2 expression during development depends strongly on the CNS areas and species analyzed. The earliest KCC2 expression in mouse CNS has been detected in hindbrain at E9.5 (Chambers *et al.*, 2009). Once started, KCC2 expression grows steeply and, upon reaching adult levels, remains mostly unchanged.

Several associations have been noted for the developmental upregulation of KCC2 expression:

1) KCC2 expression follows neuronal maturation (Li *et al.*, 2002; Wang *et al.*, 2002; Shimizu-Okabe *et al.*, 2002; Stein *et al.*, 2004; Zhang *et al.*, 2007).
2) KCC2 expression develops in a caudal-to-rostral direction (Li *et al.*, 2002; Stein *et al.*, 2004).
3) KCC2 expression develops in a ventral-to-dorsal direction

(Hubner *et al.*, 2001; Li *et al.*, 2002; Delpy *et al.*, 2008).

At least, five mechanisms have been suggested to be important for the developmental upregulation of KCC2 expression:

1) GABAergic signalling (Ganguly *et al.*, 2001; Leitch *et al.*, 2005; Simat *et al.*, 2007).
2) Glutamatergic signalling (Gulyas *et al.*, 2001;Sernagor *et al.*, 2003;Takayama & Inoue, 2006;Kanold & Shatz, 2006;Sipila *et al.*, 2009).
3) Cholinergic signalling (Liu *et al.*, 2006).
4) Cell-to-cell and/or cell-to-matrix contacts (Li *et al.*, 1998; Ludwig *et al.*, 2003).
5) Neurotrophins (Aguado *et al.*, 2003; Carmona *et al.*, 2006).

Unfortunately, the proposed mechanisms are still inconclusive and often contradicting to each other, thus impeding generation of the consensus mechanism that underlies the developmental upregulation of KCC2. For example, while several studies highlighted the role of GABAergic signalling in KCC2 developmental upregulation (Ganguly *et al.*, 2001;Leitch *et al.*, 2005;Simat *et al.*, 2007), other reports have excluded this possibility (Ludwig *et al.*, 2003;Wojcik *et al.*, 2006;Sipila *et al.*, 2009). Similarly, whereas some authors suggested the importance of glutamatergic signalling for the KCC2 upregulation during development (Gulyas *et al.*, 2001;Sernagor *et al.*, 2003;Takayama & Inoue, 2006;Kanold & Shatz, 2006;Sipila *et al.*, 2009), blocking of neither glutamatergic transmission nor action potentials was able to change KCC2 expression in dissociated neuronal cultures (Ganguly *et al.*, 2001;Ludwig *et al.*, 2003). Hypotheses explaining the KCC2 upregulation by cholinergic signalling (Liu *et al.*, 2006) or by cell-to-cell contacts (Li *et al.*, 1998; Ludwig *et al.*, 2003) have got so far little experimental confirmation. A major problem of the hypothesis that KCC2 is regulated by neurotrophins (Aguado *et al.*, 2003;Carmona *et al.*, 2006) is that spontaneous and correlated network activities, as well as maturation of GABAergic signalling are significantly changed in the TrkB-pathway affected embryos (Aguado *et al.*, 2003;Carmona *et al.*, 2003;Carmona *et al.*, 2006). Thus, it is difficult to conclude whether the observed KCC2 upregulation is a direct result of BDNF regulation or just a consequence of the abovementioned changes. In general, the apparent interconnection between the several potentially important factors makes it challenging to decipher the role of each of them in mediating the developmental KCC2 upregulation.

1.3.8 Neuronal subpopulations lacking KCC2 in adult brain

As reviewed in the previous sections, KCC2 (mRNA and protein) is expressed very broadly among adult CNS neurons. However, a few neuronal subpopulations in adult rodent brain are reported as not expressing KCC2:

1) Dorsolateral part of the paraventricular nucleus (Kanaka *et al.*, 2001).
2) Dorsomedial part of the suprachiasmatic nucleus (Kanaka *et al.*, 2001; Belenky *et al.*, 2008; Belenky *et al*., 2009).

3) Ventromedial part of the supraoptic nucleus (Kanaka *et al.*, 2001; Leupen *et al.*, 2003).
4) Reticular thalamic nucleus (Kanaka *et al.*, 2001; Bartho *et al.*, 2004).
5) Medial habenular nucleus (Kanaka *et al.*, 2001; Wang *et al.*, 2006; Kim & Chung, 2007).
6) Mesencephalic trigeminal nucleus (Kanaka *et al.*, 2001; Toyoda *et al.*, 2005).
7) Dopaminergic neurons in the substantia nigra pars compacta (Gulacsi *et al.*, 2003).

The last sections will be dedicated to the analysis of these KCC2-negative regions. An attempt will be taken to identify certain commonalities between these neuronal subpopulations lacking KCC2 expression that might help us to dissect the transcriptional mechanisms regulating the *SLC12A5* gene activity.

1.3.8.1 Paraventricular nucleus of hypothalamus

The fact that KCC2 is not expressed in the dorsolateral part of the paraventricular nucleus (PVN) was first reported by groups of Sato and Fukuda (Kanaka *et al.*, 2001). The authors analyzed the KCC2 expression in adult rat nervous system by *in situ* hybridization and found no KCC2 mRNA in the dorsolateral part of PVN. Since PVN is known to be enriched with vasopressin (VP) neurons, the authors compared the distribution of VP neurons (assessed by IHC with an anti-vasopressin antibody) with that of KCC2-positive neurons (analyzed by *in situ* hybridization with two different KCC2 probes). The VP-positive neurons, preferentially located in the dorsolateral part of PVN, were devoid of KCC2 expression (Kanaka *et al.*, 2001). However, no independent confirmation of the KCC2 absence in PVN has been obtained so far.

1.3.8.2 Suprachiasmatic nucleus

The same report also provided evidence that KCC2 was not expressed in the dorsomedial part of the suprachiasmatic nucleus (SCN) (Kanaka *et al.*, 2001). Mutually exclusive patterns of the KCC2 and VP containing neurons were observed there: VP expressing neurons, detected mostly in the dorsomedial part of the SCN, showed no hybridization signals corresponding to the KCC2 mRNA (Kanaka *et al.*, 2001).

The absence of KCC2 in the dorsomedial part of SCN was independently confirmed by the group of Pickard (Belenky *et al.*, 2008). They exploited double IHC with antibodies against KCC2 (C-terminal) and VP to show that the VP-positive neurons in the dorsomedial part of rat SCN lack KCC2 protein expression. In contrast, KCC2-ir was very prominent in the ventral and ventrolateral parts of SCN and coincided with the distribution of neurons containing the vasoactive intestinal peptide (VIP) and the gastrin-releasing peptide (GRP). Of note, VP-positive neurons, which lack KCC2, expressed strongly $GABA_B$ but not $GABA_A$ receptors. In addition, while GABA-ir was detected in most of the SCN neurons, irrespective of their peptidergic phenotype, expression of GAD65/67 and vesicular GABA transporter was significantly stronger in the ventral part of SCN (Belenky *et al.*, 2008). Next year Belenky and coauthors confirmed these results (Belenky et al., 2009). In addition, they demonstrated that KCC1, similar to KCC2, is expressed preferentially in VIP

but not in VP neurons, whereas KCC3 and KCC4 are expressed exclusively in VP neurons. The authors suggested that depolarizing GABA responses could be more likely observed in the VP but not in the VIP and GRP neurons (Belenky et al., 2009).

1.3.8.3 Supraoptic nucleus

Information about KCC2 expression in the ventromedial part of the supraoptic nucleus (SON) is more controversial. Kanaka and coauthors were not able to detect KCC2 mRNA in adult rat SON by *in situ* hybridization with two different probes corresponding to exons 9 and 24 of the *SLC12A5* gene (Kanaka *et al.*, 2001). SON, similar to PVN and SCN, is known to contain many VP neurons. Thus, Kanaka and coauthors were the first who noted the negative correlation between KCC2 and vasopressin expression. In contrast, Leupen and coworkers reported that up to 98% of the VP-positive neurons in adult mouse SON did express KCC2 (Leupen *et al.*, 2003). The reason for this controversy is unclear; both studies exploited *in situ* hybridization to detect KCC2, albeit using different probes. The probe used by Leupen *et al*. was partially homologous to the KCC1 mRNA sequence that might explain the discrepancy. Leupen and coauthors, in addition, reported that ~35% of the gonadotropin-releasing hormone (GnRH) neurons in both male and female mouse adult brain expressed KCC2 (Leupen *et al.*, 2003). However, another study, using the same IHC/*in situ* technique revealed that only 5% of the GnRH neurons express KCC2 (DeFazio *et al.*, 2002).

1.3.8.4 Reticular thalamic nucleus

Kanaka and coauthors also revealed the absence of KCC2 mRNA expression in the reticular thalamic nucleus (nRt) (Kanaka *et al.*, 2001). A more detailed analysis of the KCC2 protein expression in rat thalamus and in nRt particularly, was performed by Bartho and coauthors (Bartho *et al.*, 2004). They confirmed the lack of KCC2 in most of the nRt, but found that the dorsal and the most anterior parts of nRt still expressed KCC2 at levels similar to those in the neighbouring thalamic nuclei. To verify their results, Bartho and coworkers performed double IHC with antibodies against KCC2 and parvalbumin. They revealed that KCC2-ir and PV-ir were colocalized in the dorsal and anterior parts of nRt, but not in other nRt areas (Bartho *et al.*, 2004). The authors also noted that the KCC2 expression pattern in these parts of nRt follows the distribution of calretinin (CR) neurons (Lizier *et al.*, 1997). By using double IHC against KCC2 and CR, they confirmed this observation not only for the dorsal and anterior parts of nRt, but for the whole nRt (Bartho *et al.*, 2004).

1.3.8.5 Habenular nucleus

Three groups have so far demonstrated the lack of KCC2 expression in the medial habenular nucleus (MHb) (Kanaka *et al.*, 2001; Wang *et al.*, 2006; Kim & Chung, 2007). Kanaka and coworkers were unable to detect the KCC2 mRNA by *in situ* hybridization in the MHb, while they detected a very prominent expression in the lateral habenular nucleus (LHb). Their results were confirmed on the protein level by the group of Xu (Wang *et al.*, 2006), who exploited IHC with the KCC2 antibody from Upstate and revealed a strong KCC2-ir in the LHb, but not in the MHb. Interestingly, similar to the situation in the SCN, a very strong expression of $GABA_B$ receptors was detected in the

MHb. The $GABA_B$ receptors in MHb were functional, as detected by application of $GABA_B$-specific agonist baclofen. Another similarity to the SCN was the absence of the GABA-producing enzyme GAD65 in MHb. Nevertheless, GABA was present in MHb at levels similar to LHb because of the other GAD isoform-GAD67, which is known to be responsible for GABA synthesis in cell soma and dendrites (Wang *et al.*, 2006). Absence of KCC2, very strong expression of $GABA_B$ receptors, and prominent GABA-ir in MHb were confirmed by another study (Kim & Chung, 2007). Kim and Chung reported dual GABAergic responses in MHb: $GABA_A$ receptors mediated fast excitation, while $GABA_B$ receptors generated a strong low-threshold slow inhibition (Kim & Chung, 2007).

1.3.8.6 Mesencephalic trigeminal nucleus

Rat trigeminal nervous system consists of two sensory components: the trigeminal ganglion (TG) and the mesencephalic trigeminal nucleus (Me5). TG and Me5 contain cell bodies of primary sensory neurons, similar to the dorsal root ganglia (DRG) (Lazarov, 2002). While TG is located outside the brain, Me5 is situated in the brainstem and thus represents an exceptional case of primary sensory neurons positioned inside the brain. Importantly, Me5 originates from neural crest cells, similar to DRG (Hoover *et al.*, 2000; Lazarov, 2002), and thus it is not surprising that KCC2 mRNA was not detected in Me5 (Kanaka *et al.*, 2001). A more detailed analysis of the KCC2 mRNA in the trigeminal nervous system was performed by the group of Fukuda, and they confirmed the absence of KCC2 expression in Me5 and TG (Toyoda *et al.*, 2005).

1.3.8.7 Substantia nigra

By using a C-terminal KCC2 antibody and a tyrosine hydroxylase (TH) antibody as a marker of the dopaminergic neurons, Gulacsi and coworkers revealed two nonoverlapping patterns in the substantia nigra (SN): none of the TH-positive dopaminergic dendrites or somata were outlined by KCC2-ir, while all KCC2-positive neurons were TH-negative (Gulacsi *et al.*, 2003). The authors demonstrated that the KCC2 protein was expressed in a subset of GABAergic neurons of substantia nigra pars reticulata (SNr), identified by parvalbumin staining. Interestingly, the dopaminergic neurons of the substantia nigra pars compacta (SNc), which is devoid of KCC2 immuno-reactivity, were found to express a chloride channel ClC-2 (Gulacsi *et al.*, 2003). Gulacsi and coauthors suggested that ClC-2 and a Na^+-dependent anion exchanger (NDAE), by creating a driving force for Cl^-, might maintain hyper-polarizing GABA responses in the dopaminergic neurons of the SNc (Gulacsi *et al.*, 2003).

1.3.9 Neuronal subpopulations lacking KCC2 expression: conclusions

A strong expression of vasopressin (VP) is observed in neuronal subpopulations lacking KCC2 in the dorsolateral part of the PVN, the dorsomedial part of the SCN, and the ventromedial part of the SON (Kanaka *et al.*, 2001;Belenky *et al.*, 2008). One possible explanation for this fact might be that VP somehow downregulates KCC2 gene activity: the strongest VP expression in adult rat brain is observed in the PVN, SCN, and SON (Hallbeck *et al.*, 1999) – exactly in the areas where KCC2 is absent. Among other neuronal subpopulations expressing VP robustly

are: the anterodorsal part of the medial nucleus of the amygdala, the bed nucleus of the stria terminalis, and the nucleus of the diagonal band (Hallbeck *et al.*, 1999). However, these areas are known to express KCC2 at high levels (Kanaka *et al.*, 2001), thus, suggesting that VP downregulation of KCC2 expression is hardly probable.

Another possibility is that the lack of KCC2 expression leads to certain intracellular conditions that favour the VP gene expression. Binding sites for several activity-dependent TFs have been identified in the VP promoter: AP1, CRE, and multiple E-boxes (Grace *et al.*, 1999). Depolarizing GABA responses observed in the absence of KCC2 might lead to increased neuronal activity and as a consequence to the activation of c-FOS, CREB, and/or multiple basic helix-loop-helix (e.g. upstream stimulatory factors USF1/2) factors. The upregulation of the VP promoter by these TFs has been demonstrated previously (Burbach, 2002). In agreement with this, a substantially increased expression of c-FOS was detected in genetically modified mice expressing only 5% of the wild type KCC2 level (Woo *et al.*, 2002).

Instead of a direct link between the KCC2 and VP expression, both genes could be regulated by a common TF that activates VP and inhibits KCC2 expression in PVN, SCN, and SON. An expression pattern of such a candidate TF should be restricted to the KCC2-negative neuronal subpopulations. One possible candidate is Clock, which is strongly expressed not only in the SCN, PVN, and SON, but also in the MHb (Shieh, 2003). Clock is known to bind E-box sites in the VP promoter and to upregulate the VP gene expression (Jin *et al.*, 1999). However, it is still remains to be determined whether Clock is able to directly downregulate the KCC2 gene expression.

Three groups have also reported a strong expression of $GABA_B$ receptors in the KCC2-negative neuronal subpopulations in SCN and MHb (Wang *et al.*, 2006; Kim & Chung, 2007; Belenky *et al.*, 2008). In addition, high levels of the $GABA_B$ receptor expression were detected in adult rat brain in magnocellular neurosecretory cells of the PVN and SON (Richards *et al.*, 2005). Nevertheless, it is difficult to make further conclusions about a possible link between KCC2 and $GABA_B$ expression. Functional $GABA_B$ receptors are heterodimers, which consist of $GABA_{B1}$ (with two major isoforms $GABA_{B1a}$ and $GABA_{B1b}$) and $GABA_{B2}$ subunits (Ulrich & Bettler, 2007). Even though expression of the $GABA_{B1a}$/$GABA_{B1b}$/$GABA_{B2}$ subunits has been carefully studied (Fritschy *et al.*, 1999; Martin *et al.*, 2004; Fritschy *et al.*, 2004), these data do not allow one to conclude what parts of the adult brain possess functional $GABA_B$ signalling. Another problem is the heterogeneity of the $GABA_B$ responses: (1) modulation of synaptic vesicle priming mediated by adenylate cyclase (Sakaba & Neher, 2003), (2) inhibition of Ca^{2+} channels (Mintz & Bean, 1993), and (3) activation of K^+ channels (Luscher *et al.*, 1997). This heterogeneity significantly complicates analysis of the possible influences of $GABA_B$ signalling on the KCC2 promoter activity. More experiments are required to reveal a possible link between $GABA_B$ signalling and KCC2 gene expression.

1.4 MECHANISMS OF NEURON-SPECIFIC GENE EXPRESSION

1.4.1 Neuron-specific genes

What are the mechanisms mediating neuron-specificity of gene expression? First attempts to answer this question were undertaken about twenty years ago following characterization of several neuronal genes (Mandel and McKinnon, 1993). In the next chapter, current models and hypotheses explaining mechanisms of neuron-specific gene expression will be reviewed.

From the very beginning it is important to agree on the terminology; sometimes the available data does not clearly specify whether expression of a certain gene is indeed neuron-specific or just neural/brain-specific. Obviously, a neural pattern of gene expression does not necessarily imply neuron-specificity because of, for example, expression in glial cells. Similarly, the brain-specific expression pattern does not always imply that the gene of interest is expressed exclusively in neurons; and *vice versa*, many of neuronal genes may be expressed outside brain. Thus, to avoid uncertainties in terminology, this review will rather focus on genes that are known to be expressed predominantly in neurons and not in other cell types.

Among first genes shown to be strongly suppressed in nonneural tissues were: neuron growth-associated protein 43 (*GAP-43*) (Goslin *et al.*, 1988), superior cervical ganglion-10 (*SCG10*) (Stein *et al.*, 1988; Mori *et al.*, 1992), type II sodium channel (*NaII*) (Maue *et al.*, 1990; Kraner *et al.*, 1992), synapsin I (Ueda and Greengard, 1977; Kilimann and DeGennaro, 1985), peripherin (Thompson and Ziff, 1989), and dopamine β-hydroxylase (Ishiguro *et al.*, 1993). Nowadays, using advantages of the microarray technology, expression of many other genes is known to be neuronal and strongly suppressed outside nervous system (Wu and Xie, 2006).

1.4.2 NRSF-mediated silencing of neuronal genes in nonneuronal cells

Soon after first neuronal genes had been characterized, the question arose concerning promoter elements mediating the neuron-specificity. A classical approach to identify such elements is to clone the promoter region and to place a reporter gene under control of this region. The reporter activity of the neuronal promoter is supposed to be much weaker in nonneuronal compared to neuronal cells. If deletion of some promoter region results in a derepression of the reporter activity in nonneuronal but not in neuronal cells, then the deleted DNA fragment is the necessary repressor. Using this approach, several groups identified that deletion or mutation of a short ~20-25 bp promoter element often resulted in a strong activation of the reporter in nonneuronal cells (Kraner *et al.*, 1992; Mori *et al.*, 1992; Thompson *et al.*, 1992; Ishiguro *et al.*, 1993; Li *et al.*, 1993; Pathak *et al.*, 1994). This DNA element was named as neuron restrictive silencing element (NRSE)/repressor element-1 (RE1).

To identify TFs binding NRSE/RE1 DNA element, two groups independently screened HeLa cDNA library and cloned neuron-restrictive silencer factor (NRSF)/RE1-silencing transcription factor (REST) (Chong *et al.*, 1995; Schoenherr and Anderson, 1995). Both groups revealed that NRSF/REST is expressed at high levels in nonneuronal but not in neuronal cells. The NRSF/REST protein is

related to the Krüppel-like TF family and contains one isolated (Chong *et al.*, 1995) and 8 adjacent zinc fingers (Chong *et al.*, 1995; Schoenherr and Anderson, 1995). *In vitro* experiments confirmed that the exogenously over-expressed NRSF/REST is able to bind NRSE/RE1 and to decrease by ~10-fold the reporter activity of constructs carrying NRSE/RE1 sites (Chong *et al.*, 1995; Schoenherr and Anderson, 1995).

To identify functional NRSEs in other genes, the group of David Anderson searched through GenBank DNA sequences available at that moment using a composite NRSE consensus derived from NRSE sequences of the *SCG10*, *NaII*, synapsin I, and *BDNF* genes (Schoenherr *et al.*, 1996). This search resulted in a set of 25 genes containing putative NRSE sites with no more than two mismatches to the composite NRSE consensus. Functionality of these NRSEs was analyzed by electrophoretic mobility shift assay (EMSA) and reporter assays. Most of the revealed sites were located in neuronal genes, thus confirming the original idea that NRSE acts as a suppressor of neuronal genes in non-neuronal cells. Interestingly, in addition to the neuronal genes, this search has for the first time revealed several nonneuronal genes containing NRSE sites: adenine phosphoribosyltransferase, keratin, bovine and human P450-11β, and skeletal actin (Schoenherr *et al.*, 1996). The last case is especially interesting, since actin is known to be expressed in muscle cells in which NRSF is also expressed and in which it effectively represses the *NaII* gene.

How does NRSF inhibit expression of neuronal genes outside nervous system? Two separate corepressor complexes were identified that bind NRSF to mediate gene silencing: CoREST (Andres *et al.*, 1999) and Sin3A (Grimes *et al.*, 2000). CoREST is recruited via the C-terminal part of NRSF and this interaction requires the 9th zinc finger of NRSF to be intact; a single point mutation in this zinc finger motif is able to abolish both CoREST-NRSF complex formation (Andres *et al.*, 1999) and NRSF-mediated repression (Tapia-Ramirez *et al.*, 1997). In contrast to NRSF, CoREST is strongly expressed not only in nonneuronal but also in neuronal cells (Andres *et al.*, 1999). CoREST recruits histone deacetylases 1 (HDAC1) (You *et al.*, 2001) and 2 (HDAC2) (Ballas *et al.*, 2001), which finally convert chromatin into a repressive state to silence gene expression.

Second corepressor complex is formed via interaction of the N-terminal part of NRSF with Sin3A protein (Grimes *et al.*, 2000). Similar to the CoREST-NRSF complex, Sin3A-NRSF also recruits histone deacetylases, which are in their turn responsible for subsequent chromatin modification and gene silencing (Huang *et al.*, 1999; Naruse *et al.*, 1999; Roopra *et al.*, 2000). During development, Sin3A and NRSF are expressed broadly throughout the embryo, while CoREST exhibits a much more restricted pattern (Grimes *et al.*, 2000). This suggests that the CoREST-NRSF and Sin3A-NRSF complexes might act independently.

In addition to its ability to silence neuronal genes in nonneuronal cells via chromatin modifications, NRSF can also inhibit the RNA polymerase II transcript-tional machinery directly by interacting with TATA-binding protein (TBP) (Murai *et al.*, 2004) and with RNA polymerase II small CTD phosphatases (SCPs) (Yeo *et al.*, 2005). The last case is especially interesting, because these SCPs are known

to be expressed preferentially outside nervous system.

1.4.3 NRSF-mediated regulation of neuronal genes in neurons

In contrast to the original reports which detected NRSF only outside nervous system, several groups later on detected NRSF expression also in neuronal cells (Thiel *et al.*, 1994; Palm *et al.*, 1998; Calderone *et al.*, 2003; Zuccato *et al.*, 2003; Sun *et al.*, 2005). In agreement with this, several reports demonstrated that, in addition to its silencing role in nonneuronal cells, NRSF can also repress activity of neuronal genes in neuronal cells (Bessis *et al.*, 1997; Kallunki *et al.*, 1998; Calderone *et al.*, 2003; Wood *et al.*, 2003; Zuccato *et al.*, 2003). Similarly, a retroviral-mediated expression of the dominant negative form of NRSF in chick embryos resulted in derepression of the *SCG10*, *Ng-CAM*, and β4 tubulin genes in the infected neurons (Chen *et al.*, 1998).

Interestingly, several groups have also revealed that NRSF may act as an activator in neuronal cells (Bessis *et al.*, 1997; Kallunki *et al.*, 1998; Seth and Majzoub, 2001; Armisen *et al.*, 2002). For instance, Armisen and coauthors observed a downregulation of neuronal genes in the *Xenopus* spinal cord neurons after injection of the morpholino-modified antisense oligonucleotides and after overexpression of a dominant negative form of NRSF (Armisen *et al.*, 2002).

Thus, the above data suggest that NRSF may act not only as a silencer of neuronal genes outside nervous system but also as a modulator (activator and repressor) of neuronal genes in neurons.

1.4.4 Non-NRSF-mediated repression of neuronal genes in nonneuronal cells

The original report published by the group of David Anderson (Schoenherr *et al.*, 1996) showed that two genes (dopamine β-hydroxylase (Ishiguro *et al.*, 1993) and Na/K-ATPase α3-subunit (Pathak *et al.*, 1994)), which lack NRSE sequences, demonstrate largely a neuron-specific expression. Similar results have also been obtained for the genes encoding synapsin II (Chin *et al.*, 1994; Chin *et al.*, 1999), brain-specific angiogenesis inhibitor 1-associated protein 4 (Kim *et al.*, 2004), phytanoyl-CoA hydroxylase interacting protein (PAHX-AP1) (Kim *et al.*, 2006), and dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A) (Kim *et al.*, 2006). These data suggested that repressors other than NRSF may downregulate expression of neuronal genes in nonneuronal cells.

In agreement with this suggestion, several reports have demonstrated that deletion or mutation of functional NRSEs found in some neuronal genes does not activate expression of these genes in nonneuronal cells (Bessis *et al.*, 1997; Timmusk *et al.*, 1999). Although in one case NRSE deletion indeed resulted in the ectopic expression, this happened only in a minority of nonneuronal cells (Kallunki *et al.*, 1997). Moreover, derepression of only one neuronal gene carrying NRSE element was observed in nonneuronal cells of the knockout mice lacking NRSF protein (Chen *et al.*, 1998; Jones and Meech, 1999). The aforementioned facts conform to the idea that NRSF may not necessarily be the primary determinant of the neuron-specificity, and that other transcription factors may repress neuronal genes outside nervous system.

One of the first candidates for such role was reported by the group of Edward Ziff (Thompson *et al.*, 1992). Thompson

and coauthors identified a negative regulatory element (NRE) in the promoter of the peripherin gene; this element significantly repressed expression of the peripherin gene in undifferentiated and nonneuronal cells. The NRE-binding protein was affinity purified few years later and occurred to be a member of the CTF/NF-1 TF family (Adams *et al.*, 1995). Subsequent experiments suggested that this TF (NF1-L) is able to assemble a multiprotein complex repressing the peripherin gene expression in nonneuronal cells.

Another interesting candidate is a repressor complex formed by the activator protein 4 (AP4) and the corepressor geminin (Gem). This complex has been shown to recruit histone deacetylase 3 (HDAC3) and to silence expression of neuronal genes in nonneuronal cells (Kim *et al.*, 2006).

A new type of the repressive element has been identified in the gene encoding growth-associated protein-43 (GAP-43) (Weber and Skene, 1997). Mutation of this DNA element resulted in up to 10-fold upregulation of the GAP-43 promoter activity in nonneuronal cells, but had no effect either in cultured cortical neurons or in neuronal CAD cells. The same motif has also been identified in genes encoding synaptosomal-associated protein of 25 kDa (SNAP-25) and neuronal nitric oxide synthase (NOS) that together with GAP-43 predefined the name of the repressor motif - SNOG (Weber and Skene, 1997). It is important to note that SNAP-25 promoter has previously been shown to behave in a highly neuron-specific manner (Ryabinin *et al.*, 1995).

A strong repressor region has also been found in the promoter of the gene encoding the 5-HT1A receptor (Ou *et al.*, 2000). A detailed analysis of that region revealed that it contains at least three different repressor motifs (Albert and Lemonde, 2004). One of these motifs occurred to be NRSE; it restricts the 5-HT1A gene expression to neuronal cells. The second repressive motif, called FRE, was shown to be preoccupied by the corresponding repressor TF mostly in neuronal cells expressing 5-HT1A receptor. The FRE-binding TF was characterized by the same authors and was named FRE Under Dual repression binding protein-1 (Freud-1) (Ou *et al.*, 2003). Since Freud-1 interacts with FRE motif only in neuronal cells expressing 5-HT1A, it rather plays a role of a modulator of the 5-HT1A gene expression in response to different neuronal stimuli (Ou *et al.*, 2003). The third repressor motif, named TRE, is known to bind the TF Freud-2, a homologue of Freud-1, and to assemble a repressive complex in both neuronal and nonneuronal cells (Ou *et al.*, 2000; Hadjighassem *et al.*, 2009).

One more interesting mechanism was proposed by Ballas and coworkers to explain why neuronal genes maintain a CNS-specific pattern sometimes even after deletion or mutation of NRSE sites (Ballas *et al.*, 2005). The authors revealed that the TF MeCP2 is able to bind methylated CG dinucleotides (^{m}CpG) in promoters and to assemble a repressor complex containing Sin3, CoREST, and histone deacetylases (HDAC). Importantly, specific stimuli (e.g. membrane depolarization) can induce a rapid dissociation of MeCP2 from ^{m}CpGs that leads to the activation of neuronal promoters (Ballas *et al.*, 2005). This mechanism may be responsible, particularly, for derepression of neuronal genes during differentiation of ES cells into neural progenitors.

It has also been noticed previously that promoters of several neuronal genes contain multiple GT dinucleotide repeats (Sakimura *et al.*, 1987; Sudhof, 1990; Nedivi *et al.*, 1992; Petersohn *et al.*, 1995). Whether these GT repeats mediate repression of the neuronal genes outside CNS, is a matter for future studies.

1.4.5 Mechanisms of neuron-specific gene expression: conclusions

Neuron-specific gene expression implies a permanent inactivation of the gene activity in non-neuronal cells via multiple chromatin modifications. These modifications include DNA methylation as well as histone acetylation, methylation, phosphorylation, and ubiquitination (Li *et al.*, 2007). Among enzymes known to be important for establishing the chromatin code are: DNA methyltransferases, histone acetyltransferases and deacetylases, histone methyltransferases and demethylases, ubiquitilases, kinases and phosphatases (Kouzarides, 2007). Many of these enzymes constitute a part of different repressive complexes. For example, NRSF is able to assemble a complex containing deacetylases (e.g. HDAC1, HDAC2, HDAC4, and HDAC5), demethylases (e.g. LSD1), and methylases (e.g. G9a) (Ooi and Wood, 2007).

Thus, the repressive TFs reviewed in sections 1.4.2-1.4.4 are required to recruit the chromatin-modifying complex that in their turn silence promoters of neuron-specific genes in nonneuronal cells. A binding site for one such TF (NRSF) has been found in the promoter of the *Slc12a5* gene (Karadsheh and Delpire, 2001). A possible role of this NRSE element in mediating neuron-specific expression of the *Slc12a5* gene has been studied in paper III of the thesis and will be discussed in the subsequent chapters.

2 AIMS OF THIS STUDY

The major aim of this thesis was to study transcriptional mechanisms mediating the KCC2 gene expression *in vivo* and *in vitro.*

The specific aims were:

1. To identify the gene regulatory regions mediating the KCC2 gene transcription.

2. To clone and characterize a novel KCC2 isoform.

3. To compare the expression of the two KCC2 isoforms.

4. To study transcriptional mechanisms underlying the neuronal specificity and developmental upregulation of KCC2.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

Company names, catalogue numbers, and other information concerning chemicals exploited in the thesis can be found in the original papers (I-VI). Recipes of the solutions are described in the "Materials and Methods" of the corresponding papers.

3.1.2 Oligonucleotides

Sequences of oligonucleotides used for cloning of expression and reporter constructs as well as of *in situ* and RPA probes, quantitative and semiquantitative PCRs, mutagenesis, electrophoretic mobility shift assay (EMSA), and gel shift assay are described in the original papers (I-VI). Oligonucleotides were designed either with the "Oligo Design and Analysis Tools" software (Integrated DNA Technologies, idtdna.com) or manually with Gene Runner 3.05 (Hastings Software, Inc.). Always when possible, forward and reverse primers were chosen to be located in different exons to control the amplification of genomic DNA (except for primers used for genotyping) and their annealing temperatures were adjusted to ~60 °C. When restriction sites were included into primer sequences, some extra nucleotides were usually added to 5'-ends of the primers to increase efficiency of restriction reactions. PAGE purification was routinely performed for oligonucleotides utilized in mutagenesis, EMSA, and gel shift. Most of the oligonucleotides described in the thesis were synthesized by either Proligo (Sigma-Proligo) or Oligomer OY (Helsinki, Finland).

3.1.3 Antibodies

A list of primary antibodies exploited in this study is presented in Table 2. Three different anti-KCC2 antibodies were used that recognize KCC2 isoforms either individually (KCC2a and KCC2b antibodies) or together (KCC2pan antibody). KCC2a antibody was produced by Innovagen AB (Lund, Sweden) and first characterized by our group (paper I of this thesis). Characterization of the KCC2b antibody was reported in 2001 by Hübner and coauthors (Hubner *et al.*, 2001) long before the second isoform of KCC2 was discovered. Several groups used this antibody to study KCC2 expression not even suspecting, probably, that in reality it can detect only one of the two existing KCC2 isoforms (Hubner *et al.*, 2001; Stein *et al.*, 2004; Chudotvorova *et al.*, 2005; Blaesse *et al.*, 2006).

3.1.4 Probes for *in situ* hybridization and RPA

Sequences of *in situ* hybridization probes to detect KCC2b isoform (I), both KCC2 isoforms (III), or luciferase (III) are described in the original papers. Lengths of the probes varied from ~100 bp up to ~600 bp. Similarly, sequences of RPA probes used to detect KCC2a and KCC2b isoforms simultaneously (I) or KCC2b isoform only (III) are presented in the corresponding papers.

Table 2. List of primary antibodies used in the thesis

	Host	Source	Method	Used in
Egr4	rabbit (p)	(Zipfel *et al.*, 1997)	WB, gel shift	IV, V
GAPDH	mouse (m)	MAB374 Chemicon	WB	II
GFAP	mouse (m)	Chemicon-Millipore	ICC	II
GFP	rabbit (p)	Invitrogen	WB	II
KCC2a	rabbit (p)	(paper I)	WB, IHC, ICC, CoIP	II
KCC2b	rabbit (p)	(Hubner *et al.*, 2001)	WB, IHC	II
KCC2pan	rabbit (p)	(Ludwig *et al.*, 2003)	WB, IHC, ICC	I, II , IV, VI
Luciferase	rabbit (p)	Cortex Biochem	IHC	III
Myc-tag	mouse (m)	Sigma; 9E10	WB, CoIP	II
SPAK	rabbit (p)	(Ushiro *et al.*, 1998)	WB	II
TUJ-1	rabbit (p)	Covance Research Products	ICC	II
USF1	rabbit (p)	Santa Cruz; sc-229x	ChIP, gel shift	V
USF2	rabbit (p)	Santa Cruz; sc-862x	WB, ChIP, gel shift	V

Abbreviations: WB-Western blotting; ICC-immunocytochemistry; IHC-immunohistochemistry; CoIP-co-immunoprecipitation; ChIP-chromatin immuno-precipitation; (p)-polyclonal, (m)-monoclonal.

3.1.5 Expression and luciferase reporter constructs

Numerous expression and reporter constructs were used in the thesis. In most of these cases, CMV promoter was chosen to drive expression of cDNAs of interest. More detailed information about the expression vectors can be found in the original papers. To produce multiple reporter constructs, pGL3-Basic promoter-less vector, which contains a beetle luciferase (*Firefly*), was selected as a backbone. To normalize the *Firefly* luciferase measurements, pRL-TK vector was utilized, which contains a sea pansy (*Renilla*) luciferase. Both vectors were purchased from Promega.

3.1.6 siRNA

Two siRNA duplexes targeting Egr4 mRNA expression (catalog #16708A: ID #200866 and #200867) and one negative control siRNA duplex (catalog #4611) were ordered from Ambion (Austin, TX).

3.2 METHODS

3.2.1 Bioinformatics

3.2.1.1 Primary sequence analysis

Basic sequence processing including primer design, analysis of cloning schemes, analysis of sequencing data, prediction of molecular weights and of open reading frames for protein products – all this was performed using the Gene Runner software 3.05 (Hastings Software, Inc.).

3.2.1.2 DNA and protein sequences

Genomic, mRNA, and protein sequences for all genes mentioned in the thesis were obtained from Ensembl (ensembl.org) or from NCBI (ncbi.nlm.hih.gov) web sites. The same resources were used for searching and analyzing of EST clones.

3.2.1.3 Designing of oligonucleotides

PCR primers were routinely designed using the "Oligo Design and Analysis Tools" software (Integrated DNA Technologies, idtdna.com). This resource allows PCR primers with specified parameters and locations to be designed. It also enables to calculate accurately annealing temperatures and to perform analysis of putative hairpin structures as well as of homo- and heterodimers. IDTDNA resource takes into account the intron-exon structure of the gene and attempts to design primers in different exons. In some cases (e.g. mutagenesis of TF-binding sites and EMSA), PCR primers were designed manually using Gene Runner 3.05 (Hastings Software, Inc.).

3.2.1.4 Promoter prediction

Promoter regions were predicted with the PromoterInspector software. This program is part of the commercial package of bioinformatics tools developed by Genomatix (genomatix.de) and is able to predict eukaryotic polymerase II promoters with a high specificity (Scherf *et al.*, 2000). Other programmes (including non-commercial) that predict promoter regions can be found at (linux1.softberry.com/berry.phtml).

3.2.1.5 CpG islands prediction

CpG islands are genomic regions that are enriched in CG dinucleotides, and are frequently associated with promoters of actively transcribed genes (Larsen *et al.*, 1992). Presence of multiple CG dinucleotides indicates that these regions are usually not methylated in the genome. CpG islands were identified using CpGPlot program (ebi.ac.uk/Tools/emboss/cpgplot) with standard parameters: Window (100), Step (1), Observed /Expected (0.6), Minimum Percentage GC (50), and Length (200).

3.2.1.6 TF binding sites analysis

Analysis of TF-binding sites was performed using the MatInspector software tool (Cartharius *et al.*, 2005) that is part of the Genomatix platform. Some information on the TF binding sites was obtained via the TRANSFAC 7.0 software (gene-regulation.com).

3.2.1.7 Statistics

Statistical analysis was performed using GraphPad Prism 4.01 (VI), SSPS 15.0 (I), and Excel (III, IV, V). Student's t-test was normally used for comparison of two means.

3.2.2 Model systems

3.2.2.1 Cell cultures

Several immortalized cell lines were used in the study: Neuro2a (N2a) mouse neuroblastoma (ATCC CCL-131), C6 rat glioma (ATCC CCL-107), NIH3T3 mouse fibroblasts (ATCC CRL-1658), and HEK293 human embryonic kidney cells (ATCC CRL-1573). Of note, a recent study by Shaw and coauthors revealed that HEK293 cells express mRNA and protein of several neurofilaments (NF-M, NF-L, α-internexin), which are normally found only in neuronal cells (Shaw *et al.*, 2002). In contrast, the cells don't express filament proteins usually found in nonneuronal lines (GFAP, desmin, and the higher molecular weight keratins) (Shaw *et al.*, 2002). The authors concluded that HEK293 cells might not be typical kidney cells; moreover, they suggested that these cells cannot be used as a nonneuronal control cell line.

Standard dissociated primary cultures of rat cortical neurons (I, IV, V) and mouse hippocampal neurons (II, IV, VI) were used in the thesis. Mouse cultures were prepared from E17 mice as previously described (Banker and Goslin, 1998) with minor modifications indicated in the original papers. Density of the mouse hippocampal cultures varied from 2000-5000 cells per cm^2 in the paper IV up to 50 000 cells per cm^2 in the papers II and VI. The difference in the density is mainly explained by the fact that two different techniques for cell culturing were used: a sandwich culture technique (paper IV) and normal neuronal/glia co-cultures (papers II and V). The sandwich technique allows maintaining of a relatively low density neuronal cultures that makes it easier to analyze them by IHC. The major drawback of the sandwich technique is its relative laboriousness.

Rat cultures were prepared from E18 rats and obtained from Neuronal Cell Culture Unit (Core Facility at the Neuroscience Centre, University of Helsinki). Rat neuronal cultures were used mainly for biochemical assays requiring a sufficient amount of material for reliable analysis (Western blot, RT-PCR, luciferase assay). Density of the rat cortical neuronal cultures was kept ~200 000 cells per cm^2 (papers I, IV, V).

Solutions and conditions used for cultivating of the immortalized lines and of the primary neurons are described in the original papers.

3.2.2.2 Transgenic mice

All animal experiments were approved by the Animal Research Ethics Committee of the University of Helsinki. Target constructs were prepared by cutting vector backbone sequences off the corresponding luciferase reporter constructs, separating them in agarose gel, and subsequently purifying the restriction products with a gel extraction kit. Injection of the prepared DNA target constructs into fertilized FVB/N mouse oocytes and subsequent implantation of the oocytes into recipient pseudopregnant mice was performed by personnel of the Transgenic Unit (Core Facility at the Neuroscience Centre, University of Helsinki). For each construct, in average two to three hundred oocytes were injected, and about 150 pups were genotyped to reveal presence of the luciferase cDNA. Totally 5 *KCC2(6.8)luc*, 6 *KCC2(6.8mut)luc*, and 19 *KCC2(1.4)luc* founder lines were obtained. Out of these lines, three *KCC2(6.8)luc*, four *KCC2(6.8mut)luc*, and three *KCC2(1.4)luc* independent lines were chosen for the permanent breeding. Genotyping of the obtained offspring was performed by PCR with primers specific to the firefly luciferase cDNA sequence (5'-TGG AAC CGC TGG AGA GCA AC-3' and 5'-ACG CAG GCA GTT CTA TGA GGC-3').

3.2.3 Basic techniques

Among basic techniques used in the thesis were: restriction, rapid amplification of cDNA ends (5'RACE), agarose gel electrophoresis, transformation, transfection, sequencing, and genomic DNA isolation. Most of the methods are well described in the "Molecular cloning: a laboratory manual" by Sambrook and Russel (Sambrook and Russell, 2001). Restriction enzymes were purchased from Fermentas, Promega, and New England Biolabs. Immortalized cell lines were transfected using either Fugene 6 (Roche) or jetPEI (Polyplus Transfection) transfection reagents. Primary neuronal cultures were always transfected with

Lipofectamine 2000 (Invitrogen). All constructs were sequenced by the DNA Sequencing Service (Core facility at the Institute of Biotechnology, University of Helsinki). Isolation of genomic DNA was performed according to a previously described protocol (Laird *et al.*, 1991).

3.2.4 Expression analysis

Analysis of gene activity was performed on DNA, RNA, and protein levels. Methods utilized in the thesis for each of the levels are listed below.

3.2.4.1 DNA

Methylation status of the *SLC12A5-1b* promoter was analyzed by two different methods: bisulphite sequencing and methyl-sensitive PCR (MS-PCR). Both methods include bisulphite treatment of genomic DNA as a first step, but differ in the subsequent detection of the methylated CpG dinucleotides (sequencing versus PCR).

Electrophoretic mobility shift assay (EMSA) allows checking *in vitro* whether some part of a promoter region indeed binds certain TFs. In the thesis, EMSA was used to confirm binding of Egr4 to Egr4^{KCC2} (IV) and upstream stimulatory factors USF1/2 to E-box^{KCC2} (V). Gel shift is a modification of EMSA when a specific antibody against a TF of interest is added. Interaction of the antibody with TF increases a total molecular weight of the complex and results in its decreased mobility. EMSA and gel shift assays were performed according to the "Molecular cloning: a laboratory manual" by Sambrook and Russel (Sambrook and Russell, 2001).

Chromatin immunoprecipitation (ChIP) assesses whether a TF binding site is occupied by a corresponding TF. ChIP was performed in the thesis to probe whether USF1/2 TFs bind E-box^{KCC2} in cultured rat cortical neurons (V). The procedure was performed using ChIP assay kit (Upstate Biotechnology) according to the manufacturer's recommendations. More detailed information on the conditions and solutions used can be found in the original paper (V).

Luciferase assay is a powerful method to study promoters in several ways. First, it can be used to test whether some part of genomic DNA possesses promoter activity. This is especially important when a new promoter region is predicted by bioinformatics and requires an experimental confirmation (I, III). Luciferase assay can also be used to study how individual TF binding sites affect transcriptional activity of a certain promoter region (III, IV, V, and VI). In this case the luciferase reporter constructs bearing either intact or mutated TF sites are transfected into cells and their luciferase activity is analyzed. This method is considered to be more adequate when studying the role of a particular TF compared to a simple overexpression of that factor. TF overexpression affects simultaneously expression of many other genes which in their turn could affect promoter activity of the gene of interest. Firefly luciferase was used as the major reporter in all promoter constructs in the thesis. Dual-luciferase reporter system was used (Promega) to minimize experimental variability caused by differences in cell viability, transfection efficiency, lysate preparation, and pipetting. Dual reporter system implies that a second luciferase (Renilla) is cotransfected with the first "experimental" reporter (Firefly) for normalization. Since Firefly and Renilla luciferases use different substrates, this

allows their activities to be measured one by one from the same lysate. Detailed information on the reporter constructs used in the thesis can be found in the corresponding original papers.

3.2.4.2 RNA

RNA was isolated using RNeasy Micro (Qiagen) (I, VI), RNeasy Mini (Qiagen) (VI), NucleoSpin RNA II (Macherey-Nagel, Düren, Germany) (I), TRIzol reagent (Invitrogen) (III), and RNAwiz kit (Ambion) (I, III) according to manufacturer's recommendations. The isolated RNA was always treated with DNAse I to exclude the possibility of genomic DNA interference with subsequent RNA-based expression analyses.

Routinely up to 1 µg of total RNA was reverse transcribed in a standard volume of 20 µl using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) (III), Superscript II Reverse Transcriptase (Invitrogen) (VI), or Superscript III Reverse Transcriptase (Invitrogen) (I, VI). Except for a one special case in the paper I (see below), total RNA was reverse transcribed at 37 ºC using random primers (I, III, IV, VI). Usage of random primers allows expression of multiple target genes to be analyzed simultaneously and to be normalized to expression of certain housekeeping genes. One of the possible pitfalls of the method is the possibility that some RNA secondary structures may not be disrupted completely at 37 ºC, thus preventing an efficient reverse transcription reaction. Such a situation was described for exon-1b of the *SLC12A5* gene (I), where exon-1b tends to form strong hairpin structures (Fig. S1 in I). Usage of Superscript III Reverse Transcriptase, via its increased stability, allows overcoming this problem by conducting the reverse transcription at higher temperatures (50-55 ºC). Following the reverse transcription reaction, RNA template was always eliminated by RNAse H treatment.

One of the key methods used to quantify gene expression was real-time PCR. ABI Prism 7000 (Applied Biosystems) (I, III, IV) and CFX96 (Bio-Rad) (VI) platforms were used. In both cases the SYBR Green based reagents were exploited. cDNA samples obtained after reverse transcription were diluted 1/100 to 1/10 and amplified in triplicate using the DyNAmo Flash SYBR Green kit (Finnzymes) (I), the SYBR Green PCR Master Mix kit (Applied Biosystems) (III, IV), or the iQ SYBR Green Supermix kit (Bio-Rad Laboratories) (VI). Importantly, one of the pitfalls of the SYBR Green based quantification is that this dye may incorporate into any double stranded DNA present in a reaction. Thus, primer dimers and unspecific amplification may substantially distort the real-time PCR results. To ensure that no other products were amplified in the real-time PCR reactions, a standard melting curve analysis was performed after every run.

Ribonuclease protection assay was done using RPA kit (Ambion) (I, III) as described previously (Timmusk *et al.*, 1993). Compared to the real-time PCR quantification, RNAse protection assay is a more direct and more accurate method. This method is especially useful for a relative quantification of different splice isoforms of a gene (I), as it allows a simultaneous detection of the signals corresponding to the different isoforms on the same gel. Since RPA probes are usually labelled along their whole length,

it is important to take into account sizes of the protected fragments corresponding to the different splice isoforms. More detailed information on the design of RPA probes can be found in the original papers (I, III).

In situ hybridization was used in the original paper I to study KCC2b expression pattern as well as in the paper III to assess luciferase and KCC2total mRNA distributions. In both cases sagittal sections of fresh-frozen mouse brains were processed as described previously (Lauren *et al.*, 2003). The sections were briefly postfixed in 4% PFA and rinsed in PBS and water. Then, the sections were subsequently treated with HCl for 10 min; addition of HCl increases detection sensitivity in PFA fixed samples, possibly due to its ability to denature ribosomes, thus exposing additional target mRNAs to the probe (Ambion's Technical Bulletin #507). After that, the sections were acetylated with acetic anhydride in ethanolamine to minimize background staining, dehydrated with ethanol, and air-dried. The sections were hybridized overnight at 52°C with ^{35}S-labelled hybridization probe. Length of the probes varied from ~100 up to ~600 bp (I, III).

3.2.4.3 Protein

Several methods were used in the thesis to analyze gene activity on the protein level: Western blot, IHC, and ICC. Western blot was used to assess protein expression quantitatively (II, IV, VI) as well as qualitatively (II, V). Two other methods were mainly exploited for analysis of protein distribution in brain sections (IHC) or in cultured neurons (ICC).

Briefly, Western blot technique included a preparation of protein lysates and their subsequent separation on Tris-HCl (IV, V) or Criterion XT precast Bis-Tris (Bio-Rad) (II) polyacrylamide gels. The separated proteins were transferred onto Hybond-ECL nitrocellulose membranes (Amersham), which were subsequently hybridized with different antibodies (Table 2). Blots were developed with Amersham ECL Plus reagent (Amersham Biosciences) (I, IV, V, and VI) or with Pierce ECL Western blotting substrate (Pierce Biotechnology) (II) and visualized with Luminescent image analyzer LAS-3000 (Fujifilm). All measurements were kept in the linear range of the sensitivity of the LAS-3000 CCD camera. The detected signals were quantified with Advanced Image Data Analysis (AIDA) software (Raytest). Several precautions were taken into account during the Western blot analysis of KCC2 expression. First, it was noticed that standard heating (5 min at 95 ºC) of samples before gel loading leads to a strong aggregation of KCC2 protein, thus resulting in appearance of SDS-resistant complexes that correspond to KCC2 dimers and other higher molecular weight KCC2 complexes. Similar SDS-resistant aggregates as well as recommendations how to avoid their formation were described previously (Sagne *et al.*, 1996). Thus, the heating step was avoided always when possible. Second, it was noticed that usage of β-mercaptoethanol at high concentration (up to 10%) in the loading buffer as well as of Bis-Tris polyacrylamide gels significantly decreased formation of the aggregates. One possible explanation is that both these precautions diminish amount of disulphide bonds formed between individual KCC2 protein molecules. Addition of β-mercaptoethanol, probably, disrupts already pre-existing disulphide bonds

formed during lysate preparation. Bis-Tris system, on the other hand, has much lower pH (pH 7.2 compared to pH 9.5 for the normal Tris-HCl sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system) and thus decreases formation of the SDS-resistant aggregates during gel running by preventing other protein modifications such as deamination, alkylation, and oxidation (Sagne *et al.*, 1996). Of note, Western blot is a very versatile tool and is used in the thesis not only for analyzing protein expression but also as a part of the co-immunoprecipitation procedure (CoIP) to probe protein-protein interactions (II), to analyze KCC2 oligomerization (II), to characterize expression constructs (I) and antibodies (II). In addition, immunoblotting was used to analyze fast phosphorylation-mediated signalling (VI).

IHC and ICC methods, similar to Western blot, allow analysis of gene expression on the protein level (II, III, IV, and VI). Antibodies exploited for the immunostainings are presented in Table 2. Of note, KCC2a antibody produced a strong background in PFA-fixed brain sections and in PFA-fixed neuronal cultures, thus cold methanol was used as a fixative in all staining with this antibody. Two different protocols for immuno-staining of either 40-µm thick free-floating cryosections (III) or 16-µm thick cryosections collected on slides (II, III) are described in detail in the original papers. Cryosections and cell cultures obtained from KO mice served as a negative control. In some cases, omitting of the primary antibody was the only choice to control specificity of the IHC and ICC procedures.

sizes for their hydrated ions (Impey *et al.*, 1983; Fulton *et al.*, 1996) that predeter-

In some of the experiments a modification of the IHC and ICC methods was used that allows simultaneous detection of two different proteins in the same section or cell (II). Two variants are possible: double immunostaining using two primary antibodies either from different or from the same species. The double immunostaining with two primary antibodies from different species is very similar to the normal IHC or ICC procedures; the only difference is that two primary antibodies are added simulta-neously instead of one. The second case is more interesting. Initially, a full IHC procedure with 1st primary antibody only is performed. During this first staining the 1st primary antibody is strongly diluted and the intensity of the signal is then compensated by using the tyramine signal amplification (TSA) kit (PerkinElmer Life Sciences). Next, before 2nd primary antibody is being applied, brain sections are heated up to the boiling point in PBS for 10 min. This heating step denatures the 1st primary antibody, but presumably does not affect much the staining produced by the 1st secondary antibody. Then, the sections are stained with the 2nd primary antibody according to a normal IHC procedure. The possibility of cross-reactivity is strongly diminished by the rather high dilution of the 1st primary antibody and by the heating step. More details concerning the double immuno-staining procedures can be found in the original paper II.

3.2.5 Functional analysis

Rubidium has for a long time been used as a potassium congener (Kupriyanov & Gruwel, 2005). Both demonstrate similar mines their biochemical similarity in terms of the cationic transport. In the thesis, the

functional $^{86}Rb^{+}$ uptake assay was exploited to characterize transport properties of the KCC2a and KCC2b proteins (I). Flux experiments were performed in HEK293 cells transfected with corresponding expression constructs. Two days after the transfection, the cells were exposed to the $^{86}Rb^{+}$-containing flux solution for 3 min; such a short time interval was used to ensure that measurements were made during a linear phase. Fluxes were terminated by several washes with an ice-cold solution containing 2 mM furosemide, a known inhibitor of the KCC cotransporters. More specific information about the $^{86}Rb^{+}$ uptake assay can be found in the original paper I.

4 RESULTS AND DISCUSSION

4.1 PROMOTER REGIONS OF THE MOUSE KCC2 GENE

4.1.1 Location and structure of the *SLC12A5* gene

Identification of regulatory regions for the mouse *SLC12A5* gene was initially hampered by lack of corresponding genomic sequences. A genomic fragment (~10.3 kb) of the mouse *SLC12A5* gene, containing exons 1 to 6 and a ~1.5-kb region upstream of exon-1, was isolated (Haapa *et al.*, 1999) based on rat KCC2 cDNA (Payne *et al.*, 1996). Alignment of the rat and mouse (Ehringer *et al.*, 2001) KCC2 cDNA sequences with the aforementioned genomic fragment allowed to delineate exon-1 boundaries and to define a preliminary promoter region for the *SLC12A5* gene.

The situation was radically improved after the release of the mouse (Waterston *et al.*, 2002) and human (Lander *et al.*, 2001; Venter *et al.*, 2001) genome sequences. According to the data, the *SLC12A5* gene is located on chromosome 2 in the mouse and on chromosome 20 in the human genome. In both cases the *SLC12A5* gene lies between genes encoding for the matrix metalloproteinase 9 (MMP9) and the nuclear receptor coactivator 5 (NCOA5), which are ~45 kb apart from each other (Fig. 8). The *SLC12A5* gene was originally found to spread out over ~31 kb; intergenic regions between the *MMP9* and *SLC12A5* as well as between the *SLC12A5* and *NCOA5* genes were about 13 kb and 1 kb, respectively (Fig. 8).

Human *SLC12A5* gene was initially reported to encompass 24 exons (Song *et al.*, 2002). Nowadays 26 exons are predicted for the mouse and human *SLC12A5* genes (Ensembl Genome Browser, Release 56): exons 21 and 22 in the initially predicted 24-exon structure are now known to consist each of two exons, thus resulting in the 26-exon structure.

Interestingly, 27 exons are currently predicted by Ensembl for the rat *SLC12A5* gene: mouse exon 21 (108 bp) is split into two 54-bp exons (21st and 22nd in the rat gene). Rat exon-22 shares substantial homology to the second half of mouse exon-21 (bp 55-108). Surprisingly, rat exon-21 reveals no homology at all to the first part of mouse exon-21 (bp 1-54). On

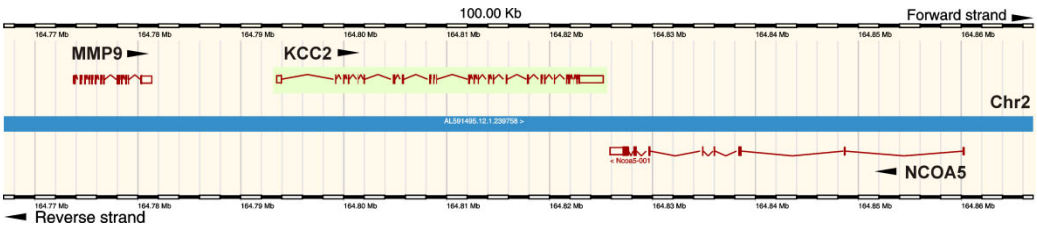

Figure 8. Genomic organization of the mouse *SLC12A5* gene. Mouse *SLC12A5* gene, encoding KCC2, is located on chromosome 2 between genes encoding for the matrix metalloproteinase 9 (MMP9) and the nuclear receptor coactivator 5 (NCOA5). The gene structure with 26 exons is shown as it had been known before identification of exon-1a. Arrowheads indicate the direction of gene transcription.

the contrary, it demonstrates a high degree of similarity (~90%) to a fragment of mouse intron-20. Thus, the current exon-intron structure of the rat *SLC12A5* gene presented by Ensembl might require additional correction.

4.1.2 Bioinformatic prediction of the *SLC12A5* promoter regions

The 45-kb mouse genomic fragment outlined by the *MMP9* and *NCOA5* genes and bearing the *SLC12A5* gene was used for a subsequent bioinformatic analysis. Since promoter regions are often associated with CpG islands (CGIs) (Antequera, 2003), the *SLC12A5* genomic locus was analyzed with the CpGPlot tool (Larsen *et al.*, 1992). Five CGIs were identified (Fig. 1A in I): CGIs II-IV – nearby the previously characterized exon-1 of the *SLC12A5* gene (Haapa *et al.*, 1999; Song *et al.*, 2002), CGI I ~7 kb upstream of exon-1, and CGI V – inside of exon 26.

The CGI I sequence was further used as a query to search a database of expression sequence tags (ESTs). Two EST clones (DA102113 and DA328785) were found that contained a part of the CGI I sequence spliced directly to exon-2 of the *SLC12A5* gene. This suggested the existence of a novel KCC2 transcript bearing a new first exon (Fig. 1A in I). This novel putative KCC2 transcript was designated as KCC2a to distinguish it from the previously characterized KCC2 isoform (Payne *et al.*, 1996; Ehringer *et al.*, 2001; Song *et al.*, 2002) which was re-designated as KCC2b. The alternative first exons of the KCC2a and KCC2b isoforms were re-named as exon-1a and exon-1b. Furthermore, the putative promoter regions upstream of exon-1a and exon-1b were designated as *SLC12A5-1a* and *SLC12A5-1b*, respectively.

The significance of CGI V is unclear given its location in the 3'UTR part of the *SLC12A5* gene (inside of exon-26). Recently, Akan and coauthors confirmed existence of the CpG island in the 3'UTR part of the *SLC12A5* gene (Akan *et al.*, 2009). They also predicted a new promoter and transcription start site (TSS) associated with this CpG island. Moreover, the authors reported a moderate H3K4me3 and a strong H3K4me2 enrichment on the spot carrying the 3′ UTR in NT2/D1 cells, thus suggesting a potentially transcribed region. Akan *et al.* proposed that this novel putative promoter might be needed for driving antisense RNA transcription to prevent the *SLC12A5* gene expression in nonneuronal cells (Akan *et al.*, 2009).

4.1.3 Experimental confirmation of the *SLC12A5* promoter regions

4.1.3.1 SLC12A5-1a promoter

To identify possible transcription start sites for the KCC2a isoform (TSS-1a), the 5'-rapid amplification of cDNA ends (5'-RACE) was performed using E15 mouse Marathon-ready cDNA. This yielded a 59 bp 5'UTR sequence followed by 121 bp of exon-1a, which encodes for the first 40 amino acids of the KCC2a isoform (Fig. 2A, in I). Similar results were obtained by using adult mouse brain RNA and GeneRacer 5'-RACE kit (Invitrogen). Analysis of EST databases revealed a single clone (DA102113) possessing the 5'UTR sequence longer than the sequence obtained by the 5'-RACE (~20 bp longer).

Interestingly, three mouse EST clones (CJ103382 [pooled tissue 7 cDNA library], CJ103009 [E15 mouse brain], and

CJ098915 [E15 mouse brain]) contained an additional 86-bp exon between exons 1a and 2. Bioinformatics predicted that inserting of this exon would produce a frame shift and abrupt the KCC2a protein peptide at the premature stop codon in exon-5. However, RT-PCR analysis of mouse brain material with a forward primer from exon-1a and a reverse primer from any of exons 2, 3, 5, and 7 did not reveal this additional 86-bp sequence between exons 1a and 2 (Fig. 1B in I, and data not shown). Moreover, no PCR products were obtained with the forward primer from exon-1a and a reverse primer from exon-1b, confirming that these exons do not splice to each other (Fig. 1B in I).

To study whether the *SLC12A5-1a* region can mediate promoter activity, a luciferase reporter was constructed, in which the luciferase was put under control of the ~1-kb genomic fragment upstream of the predicted TSS-1a. Dissociated cortical cultures were transfected at div3 and div8 with this reporter construct and the luciferase activity was measured two days later. The reporter activity of the *SLC12A5-1a* construct was ~5-fold stronger in div5 and ~15-fold in div10 cultures, when compared to the promoterless reporter (Fig. 5 in I). This suggests that the genomic fragment upstream of the predicted TSS-1a is able to mediate the neuronal transcription of the KCC2a isoform.

4.1.3.2 SLC12A5-1b promoter

A similar strategy was applied to characterize the *SLC12A5-1b* promoter. Preliminary boundaries of exon-1b were derived by aligning rat and mouse KCC2 cDNA sequences (Payne *et al.*, 1996; Ehringer *et al.*, 2001) with the corresponding genomic fragment of the mouse chromosome 2. Two independent 5'-RACE experiments pointed at the same position for a putative TSS-1b at 131 bp upstream of the initiatory methionine of the KCC2b isoform (Fig. 1a in III). Ribonuclease protection assay (RPA) confirmed the 5'RACE results: a cluster of five TSSs was detected around the -131 bp position (Fig. 1a and 1b in III). In addition, several minor TSSs were revealed by the RPA method further upstream of the major TSS-1b (Fig. 1b in III) with the most upstream site located at the position -247 bp relative to the translation start codon. After publication of paper III, additional EST clones that spread further upstream have been deposited in databases. The most 5' position for a TSS-1b is currently defined by the EST clone CJ116453, which starts at the position -420 bp relative to the translation start codon. Since no suitable initiatory methionines are present even in such a long upstream sequence of exon-1b, this does not affect the KCC2b open reading frame.

Ability of the *SLC12A5-1b* genomic fragment to mediate transcription was assessed both *in vitro* and *in vivo*. Luciferase reporter activity of the 1.4-kb *SLC12A5-1b* promoter was stronger ~13-fold in div6 and ~35-fold in div10 dissociated cortical cultures compared to the activity of the promoterless reporter (Fig. 5 in I). In Neuro-2a (N2a) neuroblastoma cells, the 1.4-kb *SLC12A5-1b* reporter was ~12-fold more active than the promoterless construct (Fig. 3 in IV). A more proximal *SLC12A5-1b* promoter fragment (0.6 kb upstream of TSS-1b) still demonstrated a strong reporter activity in cultured cortical neurons (Fig. 3 in V) as well as in N2a and NIH3T3 cell lines (Fig. 2 in V). Interestingly, even a 0.3-kb

SLC12A5-1b fragment still possessed most of the *SLC12A5-1b* 1.4-kb promoter activity in N2a cells (Fig. 3A in IV). Ability of the 1.4-kb *SLC12A5-1b* promoter to mediate transcriptional activity has also been reported in C17 neural progenitor cells (Karadsheh & Delpire, 2001). In these cells, luciferase reporter activity of the 1.4-kb *SLC12A5-1b* promoter was ~4-fold higher compared to a promoterless control reporter.

To evaluate whether the *SLC12A5-1b* promoter is transcriptionally active *in vivo*, transgenic mice were generated that carry luciferase cDNA under control of the 1.4-kb *SLC12A5-1b* genomic fragment (III). These mice displayed a strong reporter activity in CNS areas, whereas only weak or undetectable activity was detected in nonneural tissues (Table 3 in III).

4.1.4 *SLC12A5* promoter regions: conclusions

To sum up, the mouse *SLC12A5* gene (encoding KCC2) is located on chromosome 2 between the *MMP9* and *NCOA5* genes. Bioinformatics predicts two promoter regions mediating transcription of two alternatively spliced KCC2 isoforms. TSSs for both transcripts have been confirmed experimentally by RPA and/or 5'-RACE methods. Reporter experiments have confirmed that both *SLC12A5* promoters effectively drive transcription in cultured neurons and in cell lines. The *SLC12A5-1b* promoter is also active *in vivo* as demonstrated by reporter analysis in the transgenic mice (Chapter 4.4).

4.2 CHARACTERIZATION OF THE KCC2a ISOFORM

4.2.1 Cloning of the KCC2a cDNA

Transcription from the *SLC12A5-1a* promoter results in the KCC2a isoform, which in contrast to the previously known KCC2b isoform bears the alternatively spliced exon-1a. A nearly full-length rat KCC2a cDNA (including the full-length open reading frame) was amplified by two rounds of nested RT-PCR with primers located in exons 1a and 26 using total RNA isolated from the E18 rat hippocampus (Fig. 1C in I). Sequencing confirmed that the KCC2a transcript differed from the KCC2b only by the alternatively spliced first exon; all the other exons were spliced similarly in both isoforms. The absence of any additional splicing between exons 2-26 was confirmed by Blaesse *et al*., who failed to detect any aberrant KCC2 splice isoforms by two different methods (Blaesse *et al.*, 2006). The nucleotide sequence corresponding to the rat KCC2a transcript was submitted to the GenBank with an accession number EF641113.

KCC2a and KCC2b proteins are distinct only in their most N-terminal parts: exon-1a encodes a novel 40-aa N-terminal peptide instead of the 17-aa fragment encoded by exon-1b (Fig. 2A in I). Amino acid residues 1-13 in the mammalian KCC2a orthologs are highly conserved and contain a consensus binding motif for the OSR1 (oxidative stress response-1) and SPAK (Ste20-related proline alanine-rich kinase) kinases (Delpire & Gagnon, 2007) (Fig. 2B).

To study KCC2a transport properties and to assess whether KCC2a interacts with SPAK/OSR1 kinases, a KCC2a expression construct was prepared. For that, a near full-length KCC2a cDNA including the predicted 5'UTR, the whole KCC2a coding region, and a part of the 3'UTR sequence was subcloned into

pcDNA3.1 expression vector (I). Similarly, the KCC2b cDNA (a kind gift of John Payne) was cloned into the pcDNA3.1 vector that resulted in the KCC2b expression construct. Immunoblotting with the KCC2pan antibody (Ludwig *et al.*, 2003), recognizing a C-terminal peptide common in both isoforms, confirmed that each construct was able to express efficiently the corresponding KCC2 isoform in HEK293 cells. KCC2a and KCC2b products of the predicted molecular weight (~140 kDa) were detected in lysates of HEK293 cells transfected with the corresponding expression vectors but not in lysates of the mock transfected cells (Suppl. Fig. 3 in I; Fig. 1C in II).

4.2.2 KCC2a but not KCC2b can interact with SPAK in HEK293 cells

As mentioned above, the most N-terminal part of the KCC2a protein is highly conserved among mammalian orthologs and contains the OSR1/SPAK binding motif at the position 3-12 (Fig. 2B). To reveal whether SPAK can bind KCC2a, both proteins were overexpressed in HEK293 cells and a putative interaction was analyzed by CoIP. CoIP with the KCC2a antibody (II and Materials and Methods) and subsequent immunoblotting with the SPAK antibody (Ushiro *et al.*, 1998) resulted in a strong signal of the predicted size (Fig. 8A in II). This experiment has demonstrated that KCC2a can interact with SPAK in the heterologous expression system.

It has been reported recently that the KCC2b transport activity in oocytes may be modulated by SPAK (Gagnon *et al.*, 2006). Since KCC2b isoform lacks any apparent SPAK/OSR1 binding motifs, it remained unclear whether SPAK may regulate the KCC2b transport activity directly, through binding to the cotransporter in yet undetermined site, or indirectly, via modulating other kinases or phosphatases. To address this question, hemmaglutinin (HA) tagged SPAK (HASPAK) expression construct was cotransfected into HEK293 cells along with either KCC2a or KCC2b expression vectors. CoIP was first performed with the KCC2pan antibody recognizing both isoforms. Immunoblotting of the precipitated material with an HA-tag specific antibody detected a clear signal from lysates expressing KCC2a but not KCC2b isoform. A similar result was obtained when the HA antibody was used for the precipitation, while the subsequent immunoblotting was performed with the KCC2pan antibody (Uvarov *et al.*, unpublished observations).

4.2.3 Transport activity of the KCC2 isoforms in HEK293 cells

Rubidium has for a long time been used as a potassium congener (Kupriyanov & Gruwel, 2005). The biochemical similarity of Rb^+ and K^+ ions is predetermined by a similar radius size of their hydrated ions: 2.93Å for Rb^+ (Fulton *et al.*, 1996) and 2.76Å for K^+ (Impey *et al.*, 1983). $^{86}Rb^+$ was originally used as a substitute of K^+ to study the KCC2 transport properties (KCC2b isoform) in HEK293 cells (Payne, 1997). Therefore, the same $^{86}Rb^+$ uptake assay was applied to characterize the KCC2a-mediated transport activity in HEK293 cells (I). Immunoblotting confirmed a strong expression of both isoforms in HEK293 cells transiently transfected with either KCC2a (KCC2a-HEK293) or KCC2b (KCC2b-HEK293) expression vectors (supplementary Fig. 3 in I; Fig. 1C in II). The functional $^{86}Rb^+$

assay revealed ~2.5-fold higher uptakes in HEK293 cells transfected with either KCC2a or KCC2b, compared to the mock transfected cells (Fig. 6 in I). Furosemide, a known inhibitor of K-Cl cotransporters, completely eliminated this difference. Thus, in HEK293 cells, the exogenously expressed KCC2a and KCC2b isoforms demonstrated a comparable level of the furosemide-sensitive K-Cl cotransport.

Activation of K-Cl cotransport by N-ethylmaleimide (NEM) was first reported in red blood cells (Lauf *et al.*, 1984) and was confirmed later in HEK293 cells (Gillen *et al.*, 1996). The exact mechanism how NEM activates the K-Cl transporter function is unknown, but it has been suggested that NEM might inhibit certain protein kinases and simultaneously activate the membrane-associated PP2A phosphatase (Bize *et al.*, 2000). In addition, NEM is able to modify directly thiol groups in the K-Cl cotransporters (Lauf *et al.*, 1995), thus perhaps affecting their tertiary or quaternary structure. The furosemide-sensitive component of $^{86}Rb^{+}$ flux was significantly enhanced by NEM in the KCC2a-HEK293 (~2.6-fold) and KCC2b-HEK293 (~1.7-fold) cells, but not in the mock transfected cells (Fig. 6 in I). This effect of NEM was completely blocked by furosemide.

4.2.4 Characterization of the KCC2a isoform: conclusions

KCC2a cDNA was cloned following a bioinformatic prediction. The KCC2a protein differs from the previously characterized KCC2b isoform only by the most N-terminal sequence encoded by the alternatively spliced exon-1a. In contrast to KCC2b, the KCC2a protein contains the putative SPAK/OSR1 binding site. CoIP analysis shows that SPAK can bind KCC2a but not KCC2b in the heterologous expression system of HEK293 cells. In these cells, KCC2a and KCC2b isoforms mediate comparable transport activity under isotonic conditions that is furosemide-sensitive and can be further enhanced by NEM.

4.3 COMPARISON OF KCC2a AND KCC2b EXPRESSION

4.3.1 Neuronal specificity of the KCC2a and KCC2b isoforms

As it has been resumed in the "Review of the literature", KCC2 expression is substantially CNS neuron-specific. As most of the reviewed reports exploited primers, antibodies, and probes that detect simultaneously both KCC2 isoforms, the individual profiles of the KCC2a and KCC2b isoforms should also be CNS neuron-specific. Indeed, neither KCC2a nor KCC2b expression was detected by RT-PCR with primers specific for KCC2a and KCC2b in nonneural tissues (kidney, liver, and lung) at different developmental stages (E17 and P14) (Fig. 4A and 4B in I). As a positive control, spinal cord and brainstem expressed both KCC2a and KCC2b at E17; moreover, both KCC2 transcripts were detected in all brain regions and in spinal cord at P14 (Fig. 4A and 4B in I). Furthermore, no KCC2b mRNA expression was observed by RPA in the samples of adult liver and testis (Fig.1B in III), thus confirming the CNS-specific pattern for the KCC2b isoform. RPA reaction, performed with a different probe and optimized to sense lower KCC2 levels, revealed no KCC2 expression in liver and only a very weak signal in testis (Fig. 3A in I). This signal might represent a short aberrant KCC2 transcript reported previously (Williams *et al.*, 1999).

The CNS specificity of KCC2a expression has been confirmed in addition on a protein level. Immunoblotting with the KCC2a antibody detected strong KCC2a expression in spinal cord and in five brain areas, but not in six nonneural tissues (Fig.2 in II). Furthermore, double immunostainings with the KCC2a and TUJ-1 (neuronal b-tubulin) antibodies revealed a strict colocalization of the corresponding signals in dissociated hippocampal cultures, consistent with the neuron-specific pattern of KCC2a expression (Fig. 5A and 5C in II). In contrast, immunostainings did not show colocalization between KCC2a and GFAP (astroglial marker) proteins (Fig. 5B in II). Thus, multiple lines of evidence confirmed *in vivo* and *in vitro* the exclusively CNS neuronal specific patterns for both KCC2 isoforms.

4.3.2 Developmental expression of the KCC2 isoforms

As discussed in the "Review of the literature", in general KCC2 expression is upregulated during CNS development. However, since most of the data were obtained using primers, antibodies, and probes that cannot distinguish the KCC2 isoforms, additional experiments were performed to study expression profiles of the KCC2a and KCC2b isoforms individually during development. To analyze separately the KCC2 isoforms, new antibodies, probes, and primers were produced and characterized. In addition, detection methods were modified to allow an adequate comparison of KCC2a and KCC2b expression (see "Materials and Methods" part).

4.3.2.1 KCC2a expression

A relatively weak expression of KCC2a mRNA was detected by RPA in mouse whole brain samples at E14 (Fig. 3A in I). The expression was upregulated gradually between E14 and P1, and then maintained at the same level for at least up to P60. RT-PCR analysis of individual CNS regions could detect KCC2a expression in spinal cord, brainstem, and olfactory bulb, but not in cortex and hippocampus of E17 mouse embryos (Fig. 4A in I). However, KCC2a mRNA was detectable at P14 in all brain regions including cortex and hippocampus (Fig. 4B in I). More accurate real-time RT-PCR quantification revealed that at E17 the strongest KCC2a expression was in brainstem, while the cortical KCC2a level was about 5-fold lower (Fig. 4C in I). During postnatal development, the KCC2a mRNA expression decreased in brainstem ~2.5-fold, while increased in cortex ~2-fold, thus resulting at P14 in approximately similar KCC2a mRNA levels (Fig. 4C in I). These data were further confirmed on the protein level. Immunoblotting with the KCC2a antibody revealed that in brainstem and spinal cord the KCC2a protein expression was decreasing during postnatal development and was ~2-fold lower in adulthood compared to P2. In cerebral cortex, in contrast, KCC2a expression was increasing during development and was ~2-fold stronger in adult compared to P2 mice (Fig. 3B in II). Thus, both mRNA and protein data demonstrated that KCC2a expression was moderately increased during development in cortical and hippocampal areas, but decreased in brainstem and spinal cord.

4.3.2.2 KCC2b expression

The developmental profile of KCC2b expression differed radically from that for the KCC2a isoform. RPA analysis showed a steep upregulation of the KCC2b mRNA level in whole mouse brain samples between E14 and P60 (Fig. 3A in I). Another RPA experiment demonstrated similar kinetics for the KCC2b mRNA expression in whole mouse brain samples between E15 and P60 (Fig. 1B in III). These data were additionally confirmed by a semiquantitative RT-PCR that revealed no KCC2b mRNA in cortex and hippocampus at E17, but strong KCC2b expression already at P14. As a positive control, KCC2b expression in spinal cord and brainstem samples was clearly detected at both time points (Fig. 4A in I).

Real-time PCR quantification confirmed that mRNA levels of KCC2b, in contrast to KCC2a, were increased between E17 and P14 in all CNS regions analyzed (Fig. 4D in I). The KCC2b upregulation was dramatic in neocortex (~35-fold) and hippocampus (~10-fold), whereas it was rather moderate in spinal cord (~2-fold) and brainstem (~1.5-fold). The kinetics of KCC2b mRNA upregulation was additionally studied by real-time PCR in cortical and brainstem samples from 1-, 2-, and 4- week-old mice (Fig. 5C and 5F in III). Only a minor increase of KCC2b was detected in brainstem during postnatal development (Fig. 5F in III). In cortical samples, in contrast, KCC2b mRNA level increased steeply up to P14, and then remained mostly unchanged (Fig. 5C in III). The KCC2b mRNA upregulation was evident also in dissociated mouse hippocampal neurons: KCC2b mRNA level increased ~3.5-fold in between div4 and div11 (Fig. 7A in IV).

One previously published N-terminal antibody against KCC2 (Hubner *et al.*, 2001) occurred to be specific for the KCC2b isoform (Fig. 1C in II). Thus, a previous analysis of the developmental upregulation of KCC2 protein expression in mouse CNS using this antibody (Stein *et al.*, 2004) actually shows the expression of KCC2b only. In brief, a strong KCC2b protein upregulation was observed in cerebellum, hippocampus, and cortex between E15.5 and adult time points. In contrast, only moderate KCC2b protein upregulation was detected in spinal cord and brainstem (Stein *et al.*, 2004).

Interestingly, the developmental profile of the KCC2b protein expression to a large extent resembled that of the total KCC2 expression (KCC2total=KCC2a + KCC2b) in many brain areas. For instance, immunoblotting with the KCC2pan antibody, which recognizes both KCC2 isoforms, revealed a strong ~17-fold upregulation of KCC2total in cortex, but only a very moderate ~2-fold increase in brainstem during postnatal mouse development (Fig. 3A, 3C in II). Similar to cortex, a steep increase of the KCC2total expression was also observed in hippocampus and cerebellum during postnatal mouse development (Fig. 2 in IV).

4.3.2.3 Developmental shift of the KCC2a/KCC2b value

The previous data showed that the developmental profiles of the KCC2a and KCC2b isoforms differed. For example, KCC2a mRNA and protein levels decreased in brainstem and spinal cord, while KCC2b expression moderately increased in the same areas. Moreover, although both isoforms were upregulated in cerebellum, cortex, and hippocampus, the KCC2b mRNA and protein levels

grew much stronger compared to KCC2a. This suggests that the contribution of KCC2a to the total KCC2 expression would decrease during postnatal development. To measure the relative mRNA expression of the KCC2 isoforms, the RPA method was used (Fig. 3A in I). As determined by RPA, KCC2a comprised ~20% of the total KCC2 mRNA amount in whole mouse brain at E14 and remained approximately on the same level at P1. However, the KCC2a fraction decreased significantly during postnatal mouse development: at P60, only ~10% of the total KCC2 amount was represented by KCC2a in the whole brain and ~5% in cerebellum and hippocampus samples (Fig. 3A in I). The developmental down-regulation of the KCC2a mRNA fraction was confirmed by real-time PCR in several brain areas (brainstem, cortex, and hippocampus) and in spinal cord (Fig. 3B in I). The KCC2a fraction in the CNS regions was relatively high at E17, ranging from 45% in spinal cord up to 65% in cortex. The fraction was significantly smaller at P14 representing only ~15% of the total KCC2 in cortex and hippocampus, and ~30% in spinal cord and brainstem (Fig. 3B in I).

The developmental decrease in the ratio between KCC2a and total KCC2 expression was independently confirmed at the protein level. To quantify the ratio of KCC2a/KCC2total in various brain areas and at different developmental stages, protein lysates containing different amounts of KCC2a were used as standards (see Materials and Methods). The standards were loaded onto SDS-PAGE along with the brain lysates, transferred onto membrane, and immunoblotted with the KCC2a and KCC2pan antibodies (Fig. 4A in II). Standard curves (Fig. 4C in II) allowed estimation of the ratio of KCC2a to KCC2total in the analyzed brain lysates (Fig. 4B in II). A relatively high (~50-60%) KCC2a protein content was observed in brainstem, cortex, and spinal cord samples at P2. In contrast, the KCC2a/KCC2total ratio decreased significantly in adult mice representing only ~5-10% in cortex and cerebellum, and ~20% in brainstem and spinal cord (Fig. 4B in II).

In sum, both mRNA and protein data showed that the KCC2a fraction represents about half of the total KCC2 content in embryonic and neonatal brain areas but only 5-20% in adult CNS.

4.3.3 KCC2 isoforms: coexpression and heteromerization

4.3.3.1 Spatial distribution of the KCC2 isoforms in mouse brain

To study the spatial distribution of KCC2a and KCC2b in mouse brain, *in situ* hybridization was attempted. The KCC2 mRNA expression pattern, as revealed by *in situ* hybridization with a 3'UTR probe detecting both isoforms, was similar to the one reported previously (Fig. 3D in III). However, *in situ* hybridization analysis of the individual isoforms was complicated by the fact that the unique sequences for KCC2a and KCC2b are rather short and GC-rich (~75% for exon-1a and ~70% for exon-1b). The combination of these two factors significantly hampered a specific detection of the corresponding KCC2 transcripts. Anyhow, *in situ* hybridization of adult mouse brain sections with the KCC2b-specific probe (Suppl. Fig. 2 in II) revealed an expression pattern analogous to the one obtained with the 3'UTR probe (Fig. 3D in III). The strongest hybridization signals were detected in cortex,

cerebellum, and hippocampus – in those areas where the highest KCC2total protein expression and the highest KCC2b fraction were previously detected by Western blot (Fig. 4 in III).

Since *in situ* hybridization with the KCC2a antisense probe was unsuccessful (data not shown), the expression pattern of KCC2a was studied by IHC using the KCC2a-specific antibody (Fig. 1 in II). Patterns produced by the KCC2a and KCC2pan antibodies in E18 mouse brain were quite similar (Fig. 6A *versus* 6C in II). Sections from KCC2 KO mice served as specificity controls. Both antibodies produced strong signals in medulla, thalamus, and hypothalamus, while no immunoreactivity was observed in cortex and hippocampus (Fig. 6A and 6C in II). Stainings of adjacent sections of the E18 mouse brain with the KCC2a and KCC2b antibodies also did not reveal any substantial difference in the analyzed regions (Fig. 6E *versus* 6F, 6G *versus* 6H in II). At that developmental stage cortical and hippocampal areas were negative for both isoforms, whereas strong signals were detected in olfactory bulb, thalamus, and hypothalamus. Of note, although neurons in the abovementioned brain areas often coexpressed both KCC2 isoforms, the distribution of the KCC2a and KCC2b proteins in these areas was not identical (Fig. 7 in II). Preliminary results indicate that this difference between the expression patterns of the isoforms becomes more evident in the adult mouse brain (Markkanen *et al*., unpublished observations). For example, the KCC2a immunoreactivity is stronger in brainstem compared to the cerebellar cortex (Fig. 9). These results are consistent with the previous Western blot data that showed a higher expression of KCC2a (Fig. 2 in II), and a higher fraction of KCC2a in total KCC2 (Fig. 4B in II), in adult mouse brainstem compared to cerebellum. KCC2a protein expression is also much higher in hypothalamus compared to the thalamic region (Fig. 9).

To sum up, although KCC2a and KCC2b were often colocalized in perinatal mouse brain, their distributions were not always identical. Significantly different expression patterns for the KCC2 isoforms were detected in some regions of adult mouse brain.

4.3.3.2 Dimerization of the KCC2 isoforms

Homo- and heteromerization has been demonstrated previously for the CCC family members (Mckee *et al*., 2000;Moore-Hoon & Turner, 2000;Casula *et al*., 2001;de Jong *et al*., 2003;Starremans *et al.*, 2003;Simard *et al*., 2004;Blaesse *et al*., 2006;Parvin *et al*., 2007;Simard *et al*., 2007;Pedersen *et al*., 2008;Casula *et al*., 2009). Given a similar distribution of the KCC2a and KCC2b proteins in the perinatal mouse brain and their apparent colocalization in many neurons, the question arose whether the KCC2 isoforms also form hetero-oligomers. To address this question, KCC2a and KCC2b isoforms were coexpressed in HEK293 cells and their possible interaction was studied by CoIP. Protein complexes precipitated by the KCC2a antibody contained also KCC2b isoform, as detected by immunoblotting with KCC2b-specific antibody (Fig. 8A in II). Reciprocal precipitation of KCC2b and a subsequent immunodetection of the KCC2a protein showed similar results (Fig. 8B in II). As a control, no protein interaction was detected between the KCC2 isoforms when they were expressed

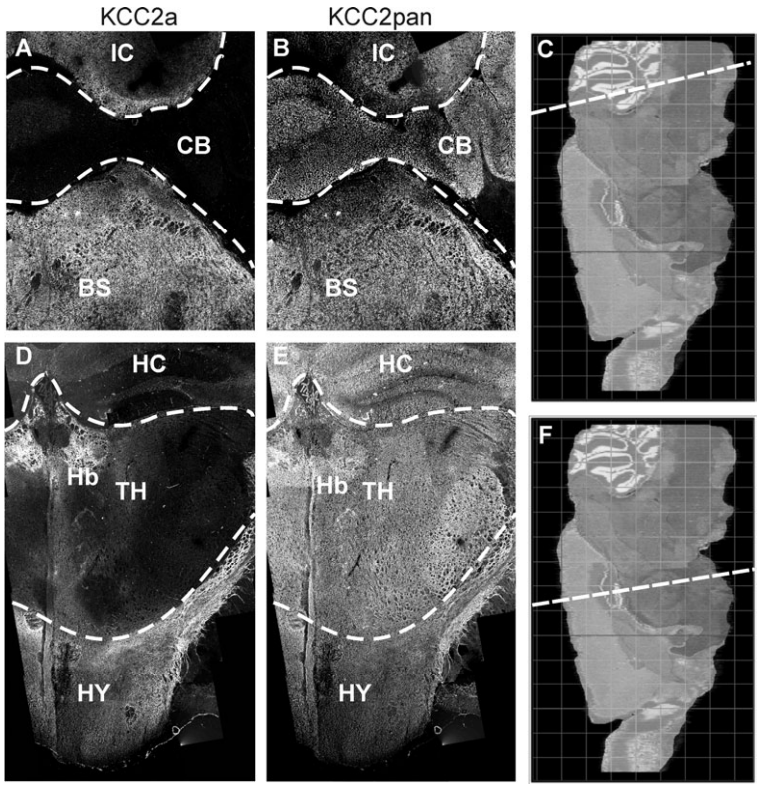

Figure 9. Areas of high KCC2a protein content in adult mouse brain. Immunostainings of adult mouse brain coronal sections demonstrate areas with a high fraction of KCC2a in the total KCC2 amount. (**A**, **B**) KCC2a immunoreactivity is significantly stronger in brainstem compared to cerebellar cortex, while levels of total KCC2 expression in these areas are similar. (**D**, **E**) Likewise, the KCC2a expression in hypothalamus is much stronger than in the thalamic region. A prominent signal detected with the KCC2pan antibody throughout thalamus implies a high content of KCC2b but not of KCC2a in this region. (**C**, **F**) Directions of cutting planes are shown. BS-brainstem, CB-cerebellum, Hb-habenula, HC-hippocampus, HY-hypothalamus, IC-inferior colliculus.

separately and the corresponding lysates were combined before the precipitation (Fig. 8A and 8B in II).

Oligomerization of the endogenously expressed KCC2a and KCC2b proteins in neonatal mouse brainstem was studied using the native perfluorooctanoate (PFO)-PAGE system. In the standard SDS-PAGE system, the endogenous KCC2 isoforms were usually observed as a ~140 kDa monomer (Fig. 1A, 1B, 2, 3A in II). In the native PFO-PAGE system, the KCC2a and KCC2b proteins in addition to monomers formed dimers (Fig. 9A in II). Importantly, no other higher molecular weight oligomers were detected. The KCC2 dimers were SDS-sensitive: brief incubation of the lysates in the presence of 0.5% SDS before loading on the PFO-PAGE disrupted the dimers (Fig. 9B in II). Formation of KCC2a-KCC2b dimers *in vivo* was further examined by CoIP. The KCC2a antibody was used to precipitate protein complexes containing KCC2a from the rat E18 brainstem lysates. Subsequent immunoblotting with the

KCC2b antibody detected KCC2b as a KCC2a-interacting partner (Fig. 9C in II). In contrast, no KCC2b-specific signals were detected when a KCC2a-unrelated antibody was used for CoIP.

Formation of the KCC2a-KCC2b oligomers was also studied in transiently transfected HEK293 cells using PFO-PAGE. In contrast to the *in vivo* situation, tri- and tetramers were observed in addition to monomers and dimers in the KCC2a-containing lysates (Fig. 8C in II). Similar set of oligomers was also present in HEK293 cell lysates overexpressing the GFP-tagged KCC2b protein. The N-terminally-fused GFP increased the molecular weight of KCC2b by ~27 kDa, resulting in the mono-, di-, tri-, and tetramers migrating slower compared to the corresponding KCC2a complexes (Fig. 8C in II). Thus, simultaneous over-expression of the KCC2a and GFP-KCC2b constructs resulted in the heterooligomeric (KCC2a)-(GFP-KCC2b) complexes with intermediate mobility (Fig. 8C in II).

To sum up, endogenous KCC2a and KCC2b isoforms are often colocalized in the perinatal rodent brain and form SDS-sensitive homo- and heterodimers. In addition to dimers, the KCC2 isoforms may form tri- and tetra-oligomers at least in the heterologous HEK293 cell system *in vitro*.

4.3.4 Expression and dimerization of the KCC2 isoforms: conclusions

The exclusively CNS neuron-specific expression of both KCC2a and KCC2b isoforms has been shown on both mRNA and protein levels. Primers, probes, and antibodies were designed to target the unique first exon sequences of the individual KCC2 isoforms, which allowed us to detect all KCC2a and KCC2b products transcribed by the *SLC12A5-1a* and *SLC12A5-1b* promoters, including those that might have been overlooked in previous assays utilizing C-terminal antibodies or 3'UTR primers and probes.

Although both KCC2 isoforms are CNS neuron-specific, their expression patterns have quite different profiles in developing rodent CNS. For example, in mice, while KCC2b expression increases in all CNS areas between E17 and P14, and especially steeply in cortex and hippocampus, KCC2a does not increase in hippocampus and even decreases in brainstem and spinal cord during this time. As a consequence, the [KCC2a/KCC2 total] value changes during postnatal development: about 50% of the total KCC2 amount is comprised of KCC2a in perinatal but only ~ 5-20% in adult mouse brain.

The increasing prevalence of KCC2b over KCC2a during development may have several implications. The ability of the KCC2a and KCC2b isoforms to form dimers suggests that the most prevalent complex in perinatal mouse brain would be KCC2a-KCC2b. On the contrary, KCC2b homodimers would be more probable in adults, especially in cortical areas that express little amounts of KCC2a. Even though the transport activities of the KCC2a and KCC2b isoforms appeared similar in HEK293 cells, this might not be the case *in vivo*. If so, even in a case when the total KCC2 amount is constant, the KCC2 activity in perinatal and adult neurons may vary significantly just because of the different composition of the KCC2 dimers.

How could the KCC2a bearing dimers possess a transport activity distinct from the KCC2b homodimers? One

possibility could be a different regulation of the KCC2a and KCC2b isoforms by SPAK. In agreement with the presence of the SPAK binding motif in KCC2a but not in KCC2b, it was KCC2a that interacted with SPAK in HEK293 cells. It has been shown previously that SPAK regulates the activity of other CCC members; moreover SPAK/OSR1 binding motifs have been identified in all KCC members except KCC1. This implies that the KCC2a-containing dimers could be regulated by SPAK in a different way than the KCC2b-containing homodimers and, as a consequence, could display different transport activities. Another possibility could be a different regulation of the KCC2a and KCC2b isoforms by other than SPAK/OSR1 kinases. For example, a putative PKA phosphorylation site has been identified that is highly conserved among mammalian KCC2a but absent from KCC2b protein sequences, (Fig. 2B in I).

4.4 MECHANISMS MEDIATING THE CNS NEURONAL SPECIFICITY OF KCC2

The data presented above demonstrate that both KCC2 isoforms are expressed in a CNS neuron-specific manner. What are the mechanisms suppressing the *SLC12A5-1a* and *SLC12A5-1b* promoters outside CNS? Since the KCC2a isoform and the corresponding *SLC12A5-1a* promoter have been discovered only recently (I), no studies have been performed so far to identify mechanisms of the KCC2a neuronal specificity. As for the *SLC12A5-1b* promoter, at least one possible explanation has been proposed: a binding site for the neuron-restrictive silencing factor/repressor element-1 transcription factor (NRSF/REST) was identified in intron-1b of the *SLC12A5* gene (Karadsheh & Delpire, 2001; Song *et al.*, 2002). NRSF is known to be strongly expressed in nonneuronal tissues, where it binds neuron-restrictive silencing element (NRSE) and efficiently suppresses transcription of NRSE-containing genes (Chong *et al.*, 1995; Schoenherr & Anderson, 1995; Ooi & Wood, 2007).

4.4.1 NRSF suppresses activity of the *SLC12A5-1b* promoter *in vitro*

The NRSE site in intron-1b of the mouse *KCC2* gene ($NRSE^{KCC2}$) is highly conserved among mammalian *SLC12A5* gene orthologs (Fig. 1 in IV). In addition, electrophoretic mobility shift assay (EMSA) has previously demonstrated that endogenous NRSF from C17 nonneuronal cells binds $NRSE^{KCC2}$ (Karadsheh & Delpire, 2001). Being artificially placed upstream of the 1.5-kb *SLC12A5-1b* sequence, $NRSE^{KCC2}$ repressed the *SLC12A5-1b* promoter activity in the C17 cells, as determined by a luciferase reporter assay.

To further assess the role of $NRSE^{KCC2}$ in neuronal specificity of the KCC2b isoform, three luciferase reporter constructs were cloned (Fig. 2A in III). Since location of NRSE relative to TSS is known to be important (Bessis *et al.*, 1997), $NRSE^{KCC2}$ was studied in its native intronic position. Therefore, the *KCC2(6.8)luc* construct contained a luciferase sequence under control of the ~6.8-kb genomic fragment comprising the 1.4-kb *SLC12A5-1b* promoter sequence, exon-1b, whole intron-1b, and a part of exon-2 (Fig. 2A in III). In comparison with the *KCC2(6.8)luc* construct, the *KCC2(6.8mut)luc* reporter lacked $NRSE^{KCC2}$ as a result of a ~340-bp deletion in intron-1b (Fig. 2A in III). A

third construct - *KCC2(1.4)luc* - contained the *SLC12A5-1b* promoter and a part of exon-1b fused to the luciferase sequence (Fig. 2A in III).

Promoter activity of these constructs was first studied in N2a cells, which don't express full-length NRSF endogenously (Palm *et al.*, 1998; Palm *et al.*, 1999). The *KCC2(6.8)luc* reporter, which contains $NRSE^{KCC2}$, was inhibited ~2-fold by exogenous overexpression of NRSF (pcDNA3-REST) (Fig. 2B in III). In contrast, the NRSF overexpression did not affect the activity of the *KCC2(6.8mut)luc* reporter, which lacks $NRSE^{KCC2}$ (Fig. 2B in III). The luciferase activity of neither *KCC2(6.8)luc* nor *KCC2(6.8mut)luc* construct was affected by overexpression of the dominant-negative form of NRSF (DN-REST) (Fig. 2B in III), which prevents binding of the full-length NRSF to NRSE (Chen *et al.*, 1998).

To sum up, $NRSE^{KCC2}$ in the intron-1b of the *SLC12A5* gene is conserved among the mammalian *SLC12A5* orthologs. Previous experiments in C17 nonneuronal cells had demonstrated that $NRSE^{KCC2}$ can interact with the endogenously expressed NRSF and suppress the transcriptional activity of the *SLC12A5-1b* promoter (Karadsheh & Delpire, 2001). Experiments presented in paper III of the thesis showed that exogenously expressed NRSF in N2a cells inhibited only partially (~2-fold) the *SLC12A5-1b* promoter. The incomplete suppression of the promoter activity in N2a cells might be explained by endogenously expressed REST4, a short form of NRSF (Palm *et al.*, 1998; Palm *et al.*, 1999). REST4 has previously been shown to interfere with the silencing activity of the full-length NRSF (Shimojo & Hersh, 2004). NRSF was not able to affect the promoter activity of the *KCC2(6.8mut)luc* reporter, in which the $NRSE^{KCC2}$ site was deleted. Thus, $NRSE^{KCC2}$ can repress the *SLC12A5-1b* promoter activity *in vitro* in the presence of either endogenously or exogenously expressed NRSF.

4.4.2 $NRSE^{KCC2}$ is not critical for the CNS neuronal specificity of *SLC12A5-1b in vivo*

To assess whether $NRSE^{KCC2}$ is sufficient to mediate the CNS neuronal specificity of the *SLC12A5-1b* promoter *in vivo*, two mouse transgenic lines were generated using the *KCC2(6.8)luc* and *KCC2(6.8mut)luc* constructs. If $NRSE^{KCC2}$ is decisive for the neuronal specificity of *SLC12A5-1b* promoter, its deletion should result in ectopic expression of the *KCC2(6.8mut)luc* reporter in nonneural tissues. In addition to these two lines, a third transgenic line, carrying the *KCC2(1.4)luc* construct, was generated and analyzed.

4.4.2.1 SLC12A5-1b promoter activity in the KCC2(6.8)luc mice

Reporter activity in the *KCC2(6.8)luc* mice occurred to be substantially CNS neuron-specific. Luciferase activity was detected in spinal cord and four brain regions (cortex, cerebellum, brainstem, and hippocampus), but neither in nonneural (liver, kidney, heart, muscle, and lung) nor in neuroendocrine (pituitary and adrenal glands) tissues (Table 1 in III). *In situ* hybridization confirmed the exclusively neuron-specific pattern (Fig. 3, column A, in III). High luciferase mRNA levels were detected in neurons of all brain regions except striatum, whereas no reporter expression was detected in glial cells of corpus callosum (Fig. 3 column A,

in III). The relatively weak hybridization signal in the striatum resembled the low expression of endogenous KCC2b mRNA in this area (Suppl. Fig. 2 in I). Of note, expression levels of total KCC2 mRNA (KCC2a+KCC2b) in the striatum and in nearby brain areas were similar as revealed by *in situ* hybridization with a probe that detects both KCC2 isoforms (Fig. 3 column D in III). This fact suggests a prevalence of KCC2a over KCC2b in the striatum.

The luciferase mRNA expression in the *KCC2(6.8)luc* mice was also very low in granular cell populations of olfactory bulb, cerebellum, and dentate gyrus (Fig. 3 column A, rows c, d, e in III). Of note, KCC2b mRNA is highly expressed in these cell populations as determined by *in situ* hybridization with the exon-1b specific probe (Suppl. Fig. 2 in I). One explanation might be that certain elements are still required to recapitulate fully the endogenous expression of KCC2b in granular cells, and that these elements are located outside the *KCC2(6.8)luc* sequence.

An apparent exception from the CNS neuron-specific expression of the *KCC2(6.8)luc* reporter was the relatively low, yet detectable (~30-50 times lower than in cortex), luciferase activity in optic nerve and peripheral sensory ganglia (trigeminal and DRG) (Table 1 in III). Lack of luciferase mRNA in the optic nerve (Suppl. Fig. 1 in III) suggested that the reporter activity in the nerve originated from neurons in the retina. To examine the cellular expression of the reporter in DRG, IHC analysis was performed using anti-luciferase antibody. A small subpopulation of luciferase-immunoreactive neurons was detected in DRGs of the *KCC2(6.8)luc* mice (Fig. 4E in III) but not of the wild type mice (Fig. 4F in III). This experiment confirmed the previous data concerning a weak KCC2 expression in DRG (Lu *et al.*, 1999; Rivera *et al.*, 1999; Ledoux *et al.*, 2006; Gilbert *et al.*, 2007; Funk *et al.*, 2008). Moreover, this result was also in agreement with the previous IHC data showing the KCC2 expression in a subpopulation of large DRG neurons (Lu *et al.*, 1999).

The KCC2 expression is also known to be weak or absent in some neuronal populations of adult mouse brain (see the "Review of the Literature"). In agreement with this, no luciferase immunoreactivity was detected in the reticulate thalamic nucleus (Fig. 4 A-D in III) and in the mesencephalic trigeminal nucleus (data not shown) of the *KCC2(6.8)luc* mice.

In sum, the 6.8-kb genomic fragment comprising the 1.4-kb *SLC12A5-1b* promoter, exon-1b, whole intron-1b, and a part of exon-2 (Fig. 2A in III) can largely recapitulate the endogenous CNS neuron-specific pattern of KCC2 expression *in vivo*.

4.4.2.2 SLC12A5-1b promoter activity in the KCC2(6.8mut)luc mice

Surprisingly, despite the $NRSE^{KCC2}$ deletion, the reporter activity in the *KCC2(6.8mut)luc* mice was still substantially CNS neuron-specific. It was strong in different brain regions and spinal cord, but undetectable in nonneural tissues (Table 2 in III). A major outcome of the $NRSE^{KCC2}$ deletion was a significant (~12-fold) upregulation of the absolute luciferase activity in the CNS areas of the *KCC2(6.8mut)luc* mice (Table 1 *versus* Table 2 in III).

Compared to *KCC2(6.8)luc*, the relative luciferase activity of the *KCC2(6.8mut)luc* construct was higher in

the peripheral ganglia (DRG and trigeminal), optic nerve, and neuroendocrine tissues (Table 2 in III) (Table 1 in III). IHC analysis confirmed that the luciferase protein was expressed in a subpopulation of large DRG neurons (III, data not shown). However, no luciferase mRNA was detected by *in situ* hybridization in optic nerve (Suppl. Fig. 1 in III). Thus, the luciferase activity in the optic nerve perhaps results from a diffusion of the luciferase protein along axons of retinal neurons. The weak luciferase activity in other peripheral and in neuroendocrine tissues of the *KCC2(6.8mut)luc* mice has probably the same explanation.

The luciferase distribution in brains of the *KCC2(6.8mut)luc* mice was analyzed by *in situ* hybridization. Similar to the situation in the *KCC2(6.8)luc* mice, the pattern of luciferase expression in the *KCC2(6.8mut)luc* mice was mostly CNS neuron-specific (Fig. 3 columns B and C in III). However, in contrast to *KCC2(6.8)luc*, the *KCC2(6.8mut)luc* construct was expressed in striatum and granular cell populations. Interestingly, the pattern of reporter activity in one of the lines (m4) did not differ from the endogenous KCC2 mRNA expression (Fig. 3 in III, compare columns C and D).
In sum, the $NRSE^{KCC2}$ deletion did not lead to ectopic reporter activity in nonneural tissues. Except for striatum and granular cells, the patterns of the luciferase expression in *KCC2(6.8)luc* and *KCC2(6.8mut)luc* mice were similar. A major result of the $NRSE^{KCC2}$ deletion was a strong upregulation of the absolute reporter activity in CNS areas (~12-fold). In combination with the preserved neuronal pattern, this upregulation suggests that $NRSE^{KCC2}$ is rather important for modulation of the *SLC12A5-1b* promoter activity in neurons than for the promoter suppression in nonneural tissues.

4.4.2.3 SLC12A5-1b promoter activity in the KCC2(1.4)luc mice

To further confirm that $NRSE^{KCC2}$ is not sufficient for suppressing the *SLC12A5-1b* promoter activity in nonneural tissues *in vivo*, *KCC2(1.4)luc* mice were analyzed. The *KCC2(1.4)luc* construct, in which the luciferase reporter is placed under control of the 1.4-kb *SLC12A5-1b* promoter and of a small part of exon-1b (Fig. 2A in III), revealed a strong reporter activity in brain and in spinal cord but not in nonneural tissues (Table 3 in III). Similar to the situation in the *KCC2(6.8mut)luc* mice, the relative luciferase activity in peripheral ganglia, optic nerve, and neuroendocrine samples was higher in the *KCC2(1.4)luc* compared to the *KCC2(6.8)luc* mice. The absolute luciferase activity in the CNS regions was about 6-fold stronger than in the *KCC2(6.8)luc* mice.

Brain sections obtained from the *KCC2(1.4)luc* mice were analyzed by *in situ* hybridization using luciferase and 3'UTR KCC2 probes. The luciferase mRNA distribution in the *KCC2(1.4)luc* mice was similar to that in the *KCC2(6.8mut)luc* mice. Granular cells and striatal neurons also expressed luciferase, but the level of expression was lower than in the nearby brain regions (data not shown).

In sum, analysis of the *KCC2(1.4)luc* mice confirmed that $NRSE^{KCC2}$ is not sufficient to mediate the CNS neuron-specific activity of the *SLC12A5-1b* promoter. The data also showed that the 1.4-kb proximal *SLC12A5-1b* promoter was largely enough for the CNS neuron-specific expression of the KCC2b isoform.

4.4.3 Transcriptional mechanisms of KCC2 neuronal specificity: conclusions

To sum up, multiple studies have previously established the CNS neuronal pattern of KCC2 expression. Experiments presented in the current thesis fully confirm these data and in addition demonstrate the CNS neuron-specificity individually for the KCC2a and KCC2b isoforms. But what are the mechanisms repressing KCC2a and KCC2b expression outside CNS? Karadsheh and Delpire have previously characterized *in vitro* a binding site for NRSF in intron-1b of the *SLC12A5* gene (Karadsheh & Delpire, 2001). The authors proposed a model, according to which NRSF binds the identified $NRSE^{KCC2}$ site and represses KCC2 expression in nonneural cells.

Cumulative *in vitro* data presented by Karadsheh and Delpire (Karadsheh & Delpire, 2001) and in this thesis demonstrate that $NRSE^{KCC2}$ could in principle be responsible for the CNS neuronal pattern of KCC2b expression. Indeed, EMSA shows that endogenous NRSF binds $NRSE^{KCC2}$ in C17 cells. Moreover, an artificial insertion of $NRSE^{KCC2}$ upstream of the 1.4-kb *SLC12A5-1b* promoter sequence inhibits by ~4-fold the reporter activity. In addition, NRSF overexpression in N2a cells suppresses the reporter activity of the *KCC2(6.8)luc* construct bearing $NRSE^{KCC2}$ but not of the *KCC2(6.8mut)luc* construct lacking this site.

Yet, *in vivo* data presented in the paper III indicate that $NRSE^{KCC2}$ is dispensable for the CNS neuronal specificity of KCC2b expression. Indeed, deletion of the $NRSE^{KCC2}$ site does not lead to ectopic expression of the *KCC2(6.8mut)luc* reporter in nonneural tissues of the corresponding transgenic mice. Moreover, the reporter activity is not observed in nonneural tissues of the *KCC2(1.4)luc* mice, in which the reporter is controlled by only the 1.4-kb *SLC12A5-1b* promoter sequence. Therefore, this fragment contains certain intrinsic repressor elements that are sufficient to prevent KCC2b expression outside CNS. Since the $NRSE^{KCC2}$ deletion increases reporter activity in *KCC2(6.8mut)luc* (~12-fold) and *KCC2(1.4)luc* (~6-fold) mice, $NRSE^{KCC2}$ may still play some role in modulating KCC2b expression in neurons.

What are those intrinsic repressor elements that silence the 1.4-kb *SLC12A5-1b* promoter in nonneural tissues? No other canonical NRSE sites were found in the 1.4-kb *SLC12A5-1b* promoter fragment. Non-canonical NRSE consensuses have recently been discovered that bind NRSF and repress gene expression (Otto *et al.*, 2007; Johnson *et al.*, 2007). These NRSE-like elements demonstrate lower binding affinity to NRSF and are less effective in gene silencing (Bruce *et al.*, 2009). However, none of the NRSE-like consensuses is present in the *KCC2(1.4)luc* sequence. Moreover, NRSF overexpression in N2a cells did not affect the reporter activity of the *KCC2(1.4)luc* construct, confirming the absence of NRSE-like sites in the 1.4-kb *SLC12A5-1b* promoter (data not shown).

Several other NRSE-containing neuronal genes, similar to *SLC12A5*, have previously failed to derepress their activity in nonneural tissues after deletion of the corresponding NRSE sites (Bessis *et al.*, 1997; Timmusk *et al.*, 1999). In a few cases when the NRSE deletion indeed resulted in ectopic expression, it happened only in a minority of nonneural cells (Kallunki *et al.*, 1997). Moreover, only a

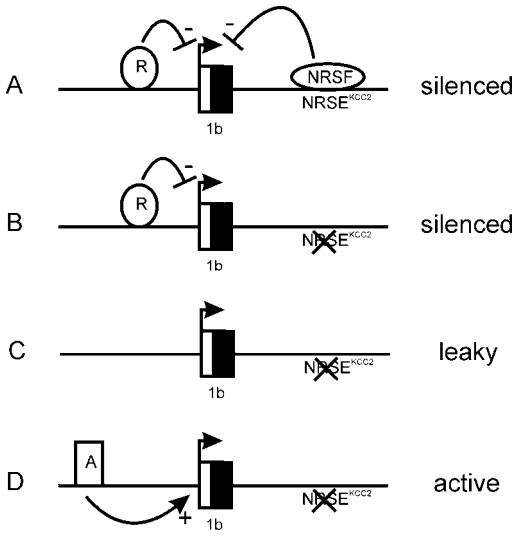

Figure 10. Additional repressor elements prevent the *SLC12A5* gene expression outside CNS in the absence of NRSE^{KCC2}. **(A**, **B)** Deletion of NRSE^{KCC2} does not derepress the *SLC12A5-1b* promoter, since a yet unidentified repressor (R) keeps the promoter silent. (**C**, **D**) Even in a case when the effects of both NRSF and R are eliminated, the expression might still be weak, because certain neuronal activator TF (A) is required to get full activation.

few neuronal genes were derepressed in nonneural tissues of NRSF KO mice (Chen *et al.*, 1998; Jones & Meech, 1999). To explain these results, David Anderson proposed a model according to which some additional repressors may assist NRSF in maintaining the neuron-specific expression (Chen *et al.*, 1998). According to this model, the NRSE^{KCC2} deletion would not derepress *SLC12A5-1b*, since a yet unidentified repressor (R) still keeps the promoter silent (Fig. 10A and 10B). Even in a case when NRSF and R are both removed, the expression might still be weak (Fig. 10C), because certain activators are required to get full activation of the *SLC12A5-1b* promoter in neurons (Fig. 10D).

What is the nature of the additional repressor(s)? One interesting mechanism was proposed for genes that lack NRSE sites, but whose promoters demonstrate a neuron-specific expression in transgenic mice (Chin *et al.*, 1999; Kim *et al.*, 2004). Kim and coauthors revealed that activator protein 4 (AP4) and the corepressor geminin (Gem) form a new functional complex that is able to silence expression of neuronal genes outside CNS (Kim *et al.*, 2006). Several putative binding sites for the AP4-Gem complex are present in the *SLC12A5-1b* promoter (data not shown).

Another mechanism to explain why neuronal genes maintain the CNS-specific pattern even after NRSE deletion was proposed by Ballas and coworkers (Ballas *et al.*, 2005). They found that TF MeCP2 is able to bind methylated CG dinucleotides (^{m}CpG) in promoters and to assemble a repressor complex containing mSin3, Co-REST, and histone deacetyl-ases. Specific stimuli (e.g. membrane depolarization) induce a rapid dissociation of MeCP2 away from ^{m}CpGs that leads to a reactivation of the promoter (Ballas *et al.*, 2005). Several CG dinucleotides, methylated in nonneural tissues but unmethylated in neurons, were identified in the *SLC12A5-1b* promoter (data not shown).

It has also been noticed previously that promoters of several neuronal genes contain multiple GT dinucleotide repeats (Sakimura *et al.*, 1987; Sudhof, 1990; Nedivi *et al.*, 1992; Petersohn *et al.*, 1995). Interestingly, similar repeats can be found in the *SLC12A5-1b* promoter (Fig. 1B in IV). Whether the GT repeats mediate repression of the neuronal genes outside CNS, is a matter for future studies.

What is the mechanism of the KCC2a repression outside CNS? As mentioned above, an influence of the $NRSE^{KCC2}$ site cannot be excluded: in some cases a single NRSE is able to repress promoters at distances up to 50 kb (Wu & Xie, 2006;Otto *et al.*, 2007), and even to silence several genes in one locus (Lunyak *et al.*, 2002). Some other, yet uncharacterized, NRSE sites may probably exist in the *SLC12A5* gene locus. In fact, at least two additional NRSE sites have been predicted: one in intron-1a (Bruce *et al.*, 2004; Wu & Xie, 2006; Johnson *et al.*, 2007) and the other in intron-7 (Lunyak *et al.*, 2002; Bruce *et al.*, 2004; Wu & Xie, 2006; Johnson *et al.*, 2007). Both NRSE sites are conserved among mammalian *SLC12A5* orthologs (Fig. 11) and located outside the KCC2 genomic fragment (Haapa *et al.*, 1999) used in paper III. Besides NRSE-mediated silencing, the *SLC12A5-1a* promoter, similar to *SLC12A5-1b*, may also have its own intrinsic repressor elements.

A new report (which appeared after the previous section had already been written) has characterized an additional NRSE element in intron-1a (NRSE-1a) of the *SLC12A5* gene (Yeo *et al*., 2009). In this report Yeo and coauthors have demonstrated that overexpression of either REST or DN-REST in cultured neurons alters the intracellular Cl^- concentration. On the one hand, this report supports the role of NRSE-mediated regulation in KCC2 gene expression. On the other hand, several uncertainties in the experiments presented in this report do not allow making a final conclusion about a possible role of the NRSE-1a element. These uncertainties, in general, can be divided in two groups: logical and methodological. Among logical worries are, for example, experiments with REST and DN-REST overexpression that do not allow assessing the impact of individual NRSE elements: it is not clear whether all three NRSE elements (in introns 1a, 1b, and 7) are required for the efficient repression of the KCC2 expression or whether any one of them alone would be enough. In addition, overexpression of REST and DN-REST in primary cortical neurons definitely affects not only the *SLC12A5* gene expression but also expression of many other genes, some of which may also regulate the inter-neuronal chloride concentration. Thus, proper controls are warranted to resolve these uncertainties.

There are also several methodological considerations. The first one concerns the design of the luciferase constructs used in the reporter assays. In some of the luciferase constructs, a part of the *SLC12A5* intron-1b was still present between exon-1b and a downstream luciferase coding sequence (Yeo *et al*., 2009). Thus, translation of the reporter, once started from the KCC2 initiatory methionine in exon-1b, would be seriously retarded (or even ceased) by numerous stop codons inside the intronic fragment, before even reaching the luciferase coding sequence. To overcome this problem, an artificial splice acceptor site has to be introduced into the 3'-end of the abovementioned intronic fragment.

Another methodological problem is found in the description of ChIP experiments presented in the report. The authors used N2a cells in their ChIP assays to demonstrate NRSF binding to the NRSE-1a element; two antibodies recognizing either N-terminus or C-terminus of NRSF revealed a strong interaction of endogenous NRSF with NRSE-1a in N2a cells. However, as it has

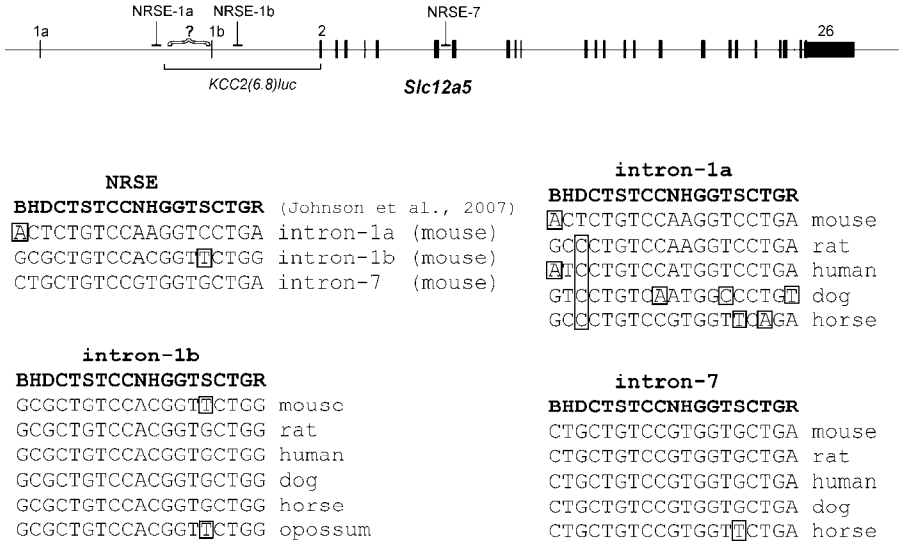

Figure 11. Location and conservation of NRSE sites in the *SLC12A5* gene. Three NRSE sites have so far been predicted for the mouse *SLC12A5* gene. NRSE elements in introns 1a (Yeo *et al*., 2009) and 1b (paper III of the thesis) were characterized previously, while analysis of NRSE site in intron-7 is warranted. This figure demonstrates schematically a location of the NRSE sites and their conservation in the mammalian *SLC12A5* orthologs. In contrast to the NRSE site in intron-1a, NRSE elements in introns 1b and 7 are highly conserved. Boxed nucleotides indicate positions that differ from the consensus. B-(G/T/C), H-(A/C/T), D-(A/G/T), S-(G/C), N-(A/G/T/C), R-(A/G).

been shown previously (Palm *et al.*, 1998; Palm *et al.*, 1999), N2a cells do not express endogenously a full-length NRSF. What this cell line indeed expresses is a truncated splice isoform REST4, which retains only five N-terminal zinc fingers, but lacks four C-terminal NRSF fingers. Thus, the positive signal observed in the ChIP experiment with the C-terminal NRSF antibody is inexplicable. Of note, as a negative control, the authors used the choline acetyltransferase (*ChAT*) gene and revealed no NRSF binding to that gene (Yeo *et al*., 2009). However, the *ChAT* gene has been shown to possess at least one functional NRSE site (Lonnerberg *et al.*, 1996) and, therefore, should be rather used as a positive control in ChIP experiments. Thus, the significance of the negative control as well as the accuracy of the whole ChIP data presented by Yeo and coauthors should be taken with great concern.

To conclude, existing data suggest that the *SLC12A5-1b* promoter has its own repressing mechanism, although some additional silencing is mediated by $NRSE^{KCC2}$. More experiments are warranted to understand mechanisms repressing the *SLC12A5-1a* promoter in nonneural cells. Conserved NRSE sites in introns 1a and 7 may further tighten expression of both isoforms, converting *SLC12A5* into one of the most neuron-specific genes in mammalian CNS (Wu & Xie, 2006).

4.5 PROMOTER MECHANISMS MEDIATING DEVELOPMENTAL UPREGULATION OF THE KCC2 EXPRESSION

Multiple reports revealed a steep up-regulation of KCC2 expression in developing rodent brain (Chapters 1.3.6; 1.3.7). However, most of the data was obtained using primers, antibodies, and probes that cannot distinguish the KCC2 isoforms. In the current thesis, the expression patterns of the KCC2a and KCC2b isoforms were assessed individually (Chapter 4.3.2). It has been established that it is KCC2b expression that increases dramatically in cortex, hippocampus, and cerebellum postnatally, while KCC2a expression changes moderately.

Reporter activity in the *KCC2(6.8)luc*, *KCC2(6.8mut)luc*, and *KCC2(1.4)luc* transgenic mice largely recapitulated the CNS neuronal pattern of endogenous KCC2b expression. In addition, the reporter activity in *KCC2(6.8)luc* (Fig. 5A, 5D in III) and *KCC2(6.8mut)luc* (Fig. 5B, 5E in III) mice followed the developmental profile of the endogenous KCC2b (Fig. 5C, 5F in III). The reporter expression increased dramatically in cortical (Fig. 5A, 5B in III) but not in brainstem samples (Fig. 5D, 5E in III). Therefore, the *SLC12A5* genomic fragment, present in the *KCC2(6.8)luc* construct, is largely enough for the developmental upregulation of KCC2b expression.

What are these *SLC12A5-1b* intrinsic elements that enhance the promoter activity in postnatal rodent brain? One possibility may be that the *SLC12A5-1b* promoter is induced by some activator TFs, whose expression is also upregulated during development (Fig. 10). Another possibility might be that the developmental upregulation is caused by a general chromatin remodelling and by subsequent derepression of the whole *SLC12A5-1b* locus. The transgenic mouse approach (Fig. 5 in III), when the reporter constructs are incorporated into the genome, does not allow to discriminate between the chromatin derepression and the action of transcriptional activators. Thus, the reporter activity was first evaluated in cultured cortical neurons transiently transfected with the corresponding promoter construct (Fig. 5 in I). The luciferase activity of the *KCC2(1.4)luc* reporter in div6 neurons was ~13-fold stronger compared to the activity of the promoterless construct, and further increased by ~2.5-fold at div10 (Fig. 5 in I). Similar results were recently reported by Yeo and coauthors, who observed a strong developmental upregulation of the (-1649/+49) *SLC12A5-1b* reporter activity in primary cortical neuronal cultures (Yeo *et al*., 2009). In comparison with the 1.4-kb *SLC12A5-1b* construct, the (-1649/+49) reporter spreads ~0.3 kb further upstream of the known TSS-1b, but similarly does not contain NRSE elements. Thus, mechanisms other than the general chromatin derepression contribute to the developmental upregulation of the 1.4-kb *SLC12A5-1b* promoter.

To identify TFs that possibly regulate KCC2b expression, a multiple alignment was performed for the mouse, rat, human, and chimpanzee genomic sequences corresponding to the 1.4-kb *SLC12A5-1b* promoter and proximal intron-1b region. Ten TF binding sites were found to be highly conserved in all four species: by two sites for specificity protein 1 (Sp1) (Kadonaga *et al.*, 1987) and activating enhancer-binding protein (AP2) (Williams & Tjian, 1991); by one site for AP1 (Lee

et al., 1987), myocyte-enhancing factor 2 (Mef2) (Gossett *et al.*, 1989), early growth response 4 (Egr4) (Swirnoff & Milbrandt, 1995), neuron restrictive silencing factor (NRSF) (Schoenherr *et al.*, 1996), and Block (Collart *et al.*, 1991); and E-box site (Sawadogo, 1988), which is known to bind numerous basic helix loop helix (bHLH) TFs (Fig. 1A, 1B in IV). Eight out of the ten sites belong to the (-415/+9) region, previously predicted to outline a proximal *SLC12A5-1b* promoter (III). Five out of the ten binding sites have so far been characterized by our group: NRSE (III), Egr4 site (IV), E-box (V), and two Sp1 sites (unpublished observations). The revealed TF binding sites, as well as the previously described CpG islands CGI's II-IV and TSSs corresponding to the *SLC12A5-1b* promoter are shown in Fig. 12. Characterization of the $NRSE^{KCC2}$ site has been explained in the previous chapter. The role of the E-box and Egr4 binding sites in the developmental upregulation of KCC2b expression will be analyzed below.

4.5.1 Regulation of the *SLC12A5-1b* promoter by Egr4 transcription factor

Egr4 belongs to the family of early growth response (Egr) transcription factors, which embraces four members: Egr1 [alias nerve growth factor-induced-A (NGFI-A), Krox-24, zinc finger-interacting 268 (zif268), Tis8, and ZENK], Egr2 [alias Krox20], Egr3 [alias Pilot], and Egr4 [alias NGFI-C]. All Egr members contain three zinc fingers, which interact with a 9-bp GC-rich core sequence. Three additional nucleotides that surround the 9-bp core (Fig. 5A in IV) add specificity for recognition of the binding site by different Egr members (Swirnoff & Milbrandt, 1995).

Several lines of evidence suggest that Egr4 might regulate KCC2 expression. Egr4 mRNA expression, similar to KCC2, has been shown to display a neural specific pattern and to be upregulated during the postnatal development (Crosby *et al.*, 1992). The "Review of the literature" has determined that neuronal activity and neurotrophins are among putative factors regulating KCC2 expression. Similarly, Egr4 expression is robustly induced by neurotrophins and by neuronal activity (Crosby *et al.*, 1991; Crosby *et al.*, 1992; Honkaniemi & Sharp, 1999).

4.5.1.1 Characterization of the Egr4 binding site: N2a cells

To characterize the Egr4 binding site found in the *SLC12A5-1b* promoter ($Egr4^{KCC2}$), a series of reporter constructs was used (Fig. 3 in IV); some of the constructs lacked the $Egr4^{KCC2}$ site. Luciferase activity was analyzed in N2a neuroblastoma cells transiently transfected with the corresponding plasmids. The basal activity of the 1.4-kb *SLC12A5-1b* promoter construct *KCC2(-1398/+42)* was ~12-fold stronger compared to the activity of the promoterless *pGL3-Basic* construct (Fig. 3A in IV). Deleting of a substantial part of the promoter did not change much the basal reporter activity: *KCC2(-309/+42)* and *KCC2(-180/+42)* constructs expressed luciferase still ~10-fold stronger than *pGL3-Basic*. However, these constructs were differentially induced by exogenously overexpressed Egr4 (Fig. 3B in IV). Each of the constructs containing the Egr4 site was strongly (15-20 times) upregulated by Egr4 but not by Egr1, another member of the Egr family that

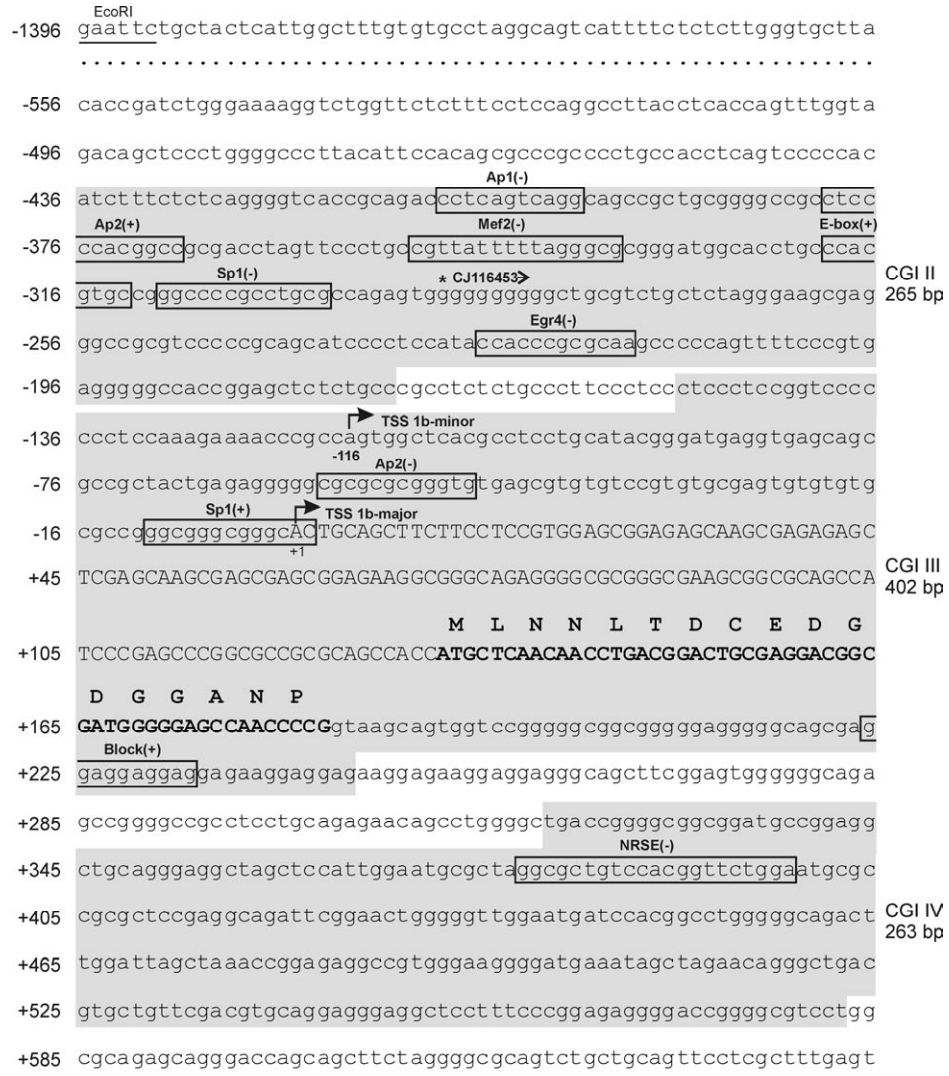

Figure 12. Summary of the bioinformatical and experimental data including CpG islands, TSSs, and TF binding sites in the *SLC12A5-1b* promoter and proximal intron-1b regions. Three CpG islands (CGI's II-IV, marked in grey) were revealed via bioinformatical analysis in the *SLC12A5-1b* promoter and proximal intron-1b regions. Ten putative TF binding sites and their locations on either the sense (+) or antisense (-) DNA strands are shown (boxed). Two transcription start sites (TSS-1b major and TSS-1b minor, marked with arrows) were identified previously for the KCC2b transcript by RNAse protection assays, 5'RACE experiments, and a subsequent bioinformatical analysis. The expressed sequence tag clone farthest upstream (position -288 bp relative to TSS-1b major) that is homologous to the KCC2b transcript (CJ116453) is marked with asterisk. Intron and promoter regions are presented in lowercase letters, exon-1b sequence appears in capital letters, and coding parts of the exons are highlighted in bold. The amino acid translation of the coding part of exon-1b is shown above the corresponding nucleotide sequence.

binds a different consensus sequence (Swirnoff & Milbrandt, 1995). No upregulation was observed for the *KCC2(-180/+42)* construct, which lacks Egr4^{KCC2} (Fig. 3B in IV). To test the possibility that Egr1 binds Egr4^{KCC2} but for some reason cannot activate the *SLC12A5-1b* promoter, increasing amounts of Egr1 expression plasmid were cotransfected along with a constant amount of Egr4 (Fig. 3C in IV). However, instead of the expected downregulation of the reporter activity (dominant negative effect of Egr1), a moderate upregulation was detected, thus indicating that Egr1 does not compete with Egr4 for Egr4^{KCC2}.

The previous experiments did not exclude the possibility that Egr4 activates the *SLC12A5-1b* promoter indirectly via regulating expression of other TFs, which in their turn affect the promoter. To test whether Egr4 can bind directly Egr4^{KCC2}, EMSA was performed. N2a cells were transfected with the Egr4 expression construct and nuclear extracts were incubated with a radioactively labelled double-stranded DNA oligonucleotide carrying the Egr4^{KCC2} sequence (Fig. 4B in IV). Three specific DNA-protein complexes were detected (Fig. 4A in IV). However, the only DNA-protein complex, whose formation was effectively prevented by unlabelled intact Egr4^{KCC2} but not by unlabelled mutant Egr4^{KCC2} oligonucleotides, was complex I (Fig. 4A in IV). In addition, Egr4 antibody was able to supershift complex I but failed to supershift two other complexes. Thus, EMSA and the supershift assay demonstrated that Egr4 can bind the Egr4^{KCC2} element.

To ensure that the Egr4-induced upregulation of the *SLC12A5-1b* promoter in the N2a cells was indeed mediated via the Egr4^{KCC2} site, this element was mutated and luciferase activity of the reporters with intact and mutant Egr4^{KCC2} sites was compared. Mutation of Egr4^{KCC2} strongly decreased ability of Egr4 to upregulate the proximal *SLC12A5-1b* promoter (Fig. 5B in IV). However, this mutation was not able to prevent completely the Egr4-induced upregulation. One possible explanation could be the presence of less stringent Egr4-like binding sites in the proximal promoter. The proximal promoter was reanalyzed and one such Egr4-like site was found. Its sequence differed from the Egr4 consensus in positions -1 and +4; moreover, it was not conserved in the *SLC12A5-1b* promoters of other mammals. Mutation of this site did not alter the reporter activity (data not shown). So, at least half of the 15-fold upregulation of the reporter activity in N2a cells is mediated by Egr4 indirectly by regulating expression of other TFs controlling the *SLC12A5-1b* promoter.

To test whether Egr4^{KCC2} site is sufficient for the Egr4-mediated induction of the *SLC12A5-1b* promoter activity, two copies of the Egr4^{KCC2} sequence were inserted into the *KCC2(-180/+42)* reporter construct, which previously failed to demonstrate the Egr4-mediated upregulation (Fig. 3B in IV). The new construct [*KCC2(-180/+42)2xEgr4(-)*], carrying the Egr4^{KCC2} duplex site in the same orientation as the original element (on the reverse DNA strand), was strongly induced by Egr4 (Fig. 5C in IV). Surprisingly, similar construct [*(KCC2(-180/+42)2xEgr4(+)*], carrying the same duplex but in the opposite orientation (on the forward DNA strand), was not induced by Egr4 (Fig. 5C in IV). It has been shown previously that members of Egr family

bind their recognition sites as monomers in an anti-parallel configuration (Pavletich & Pabo, 1991). Thus, the relative orientation of Egr4 might be important for its interaction with other TFs. Such an interaction between Egr members and certain TFs has previously been shown to be important for synergistic activation of promoter activity (Decker *et al.*, 2003). Thus, the $Egr4^{KCC2}$ sequence in the context of the proximal *SLC12A5-1b* promoter and in the correct orientation is able to mediate the Egr4-induced upregulation of promoter activity.

Usage of Egr dominant-negative isoforms has proved to be an efficient approach to analyze mechanisms by which Egr family members regulate gene expression (Levkovitz *et al.*, 2001; Roberts *et al.*, 2005). To study further the role of $Egr4^{KCC2}$ in the regulation of endogenous KCC2b expression, a dominant negative variant of Egr4 (DN-Egr4) was produced (Fig. 6A in IV). It retained the DNA-binding part of Egr4, but lacked the N-terminal activator domain. Overexpression of DN-Egr4 had no effect on the basal activity of the proximal *SLC12A5-1b* promoter construct, but effectively inhibited the Egr4-mediated induction of the same construct (Fig. 6B in IV).

4.5.1.2 Characterization of the Egr4 binding site: cultured neurons

To test whether Egr4 can also regulate endogenous KCC2b expression, a model of cultured hippocampal neurons was used. In this model both KCC2b and Egr4 mRNA levels were found to be strongly upregulated during the culture maturation (Fig. 7A in IV). Overexpression of DN-Egr4 significantly downregulated KCC2 expression as detected by immunostaining with the KCC2pan antibody (Fig. 7B, 7C in IV). Thus, DN-Egr4 was able to downregulate the endogenous KCC2 expression in cultured neurons presumably by preventing Egr4 binding to the $Egr4^{KCC2}$ site.

According to the previous data, KCC2b and Egr4 mRNA levels increased steeply in cultured hippocampal neurons between div4 and div11 (Fig. 7A in IV). Thus, the maximal effect of the Egr4 overexpression might be expected at early time points, when the endogenous expression of Egr4 is low. On the contrary, DN-Egr4 should have a stronger effect in matured cultures, when the endogenous Egr4 level is high. Indeed, overexpression of Egr4 increased the *KCC2(-309/+42)* reporter activity at div5, when the endogenous Egr4 expression is relatively low (Fig. 8A in IV), but had no effect at div10 (Fig. 8B in IV), when it is already relatively high (Fig. 7A in IV). An opposite situation was observed in a case of the DN-Egr4 overexpression: significant downregulation of the *KCC2(-309/+42)* construct activity was achieved at div10, when endogenous Egr4 expression is high, but not at div5, when the promoter regulation by the endogenous Egr4 is presumably minor (Fig. 8A, 8B).

One possible drawback of the DN-Egr4 approach is the relatively low transfection efficacy into mature cultured neurons that prevents efficient suppression of the Egr4-mediated regulation. If true, the observed ~30% downregulation of the endogenous KCC2b expression (Fig. 7A in IV) and the reporter activity of the *KCC2(-309/+42)* construct (Fig. 8B in IV) might represent a significant under-estimation of the Egr4 contribution to the *SLC12A5-1b* promoter regulation. Another way to assess the Egr4 impact is to affect

the endogenous Egr4 level by RNA interference (RNAi)-mediated knockdown. Two different anti-Egr4 siRNA duplexes decreased the luciferase activity of the *KCC2(-309/+42)* reporter by ~35% and ~25%, correspondingly (Fig. 8C in IV). Thus, the attenuation of the endogenous Egr4 level by RNAi and the prevention of the Egr4 binding to $Egr4^{KCC2}$ by DN-Egr4 significantly downregulated activity of the *SLC12A5-1b* proximal promoter region.

To assess a role of $Egr4^{KCC2}$ in the developmental upregulation of the *SLC12A5-1b* proximal promoter, the reporter activity of the *KCC2(-309/+42)* construct was compared to that of the [$KCC2(-309/+42)^{Egr4mut}$] construct, in which this site was mutated. Being analyzed in cultured hippocampal neurons at div5 and div10, the reporter activities of the *KCC2(-309/+42)* and $KCC2(-309/+42)^{Egr4mut}$ constructs increased 4.2-fold (±0.2) and 2.7-fold (±0.3), respectively ($p<0.05$; Student's *t* test). Thus, the mutation of $Egr4^{KCC2}$ significantly affected (~35%) the developmental upregulation of the *SLC12A5-1b* promoter activity.

4.5.1.3 $Egr4^{KCC2}$ is required for the BDNF-mediated upregulation of the SLC12A5-1b activity in immature neurons

Previous studies have indicated that the BDNF-TrkB signalling pathway might play an important role in the developmental upregulation of KCC2 expression (Aguado *et al.*, 2003; Carmona *et al.*, 2006). It has also been known that expression of Egr family members can be rapidly induced by neurotrophins (Crosby *et al.*, 1991; Harada *et al.*, 2001; Roberts *et al.*, 2005; Roberts *et al.*, 2006; Calella *et al.*, 2007). Thus, could it be that BDNF increases expression of Egr4 that in its turn via $Egr4^{KCC2}$ activates the *SLC12A5-1b* promoter?

To test this hypothesis, dissociated hippocampal neurons cultured for 5-6 days *in vitro* were treated with BDNF (50 ng/ml). The ERK pathway was strongly activated already 5 min after the BDNF application (Fig. 3A, 3B in VI). Moreover, Egr4 mRNA expression increased ~20-fold 2h after BDNF application (Fig. 3C in VI). Both effects were substantially blocked by a specific MEK inhibitor U0126, thus suggesting that the ERK pathway is involved into the BDNF-mediated induction of Egr4 expression (Fig. 3A-3C in VI).

The *KCC2(-309/+42)* reporter construct was used to study whether the BDNF-induced increase in Egr4 expression affects *SLC12A5-1b* promoter activity (Fig. 4A in VI). Indeed, the luciferase activity of the reporter was significantly increased after brief treatment of hippocampal cultures with BDNF (Fig. 4B in VI). The effect of BDNF on the reporter activity was blocked by inhibition of TrkB with K252a and of MEK with U0126, but not by inhibition of sodium channels with TTX (Fig. 4B in VI).

The $KCC2(-309/+42)^{Egr4mut}$ reporter, in which the $Egr4^{KCC2}$ site is mutated (Fig. 4A in VI), was further used to study the BDNF-mediated upregulation of the *SLC12A5-1b* promoter. Compared to the *KCC2(-309/+42)* construct, mutation of $Egr4^{KCC2}$ decreased basal promoter activity (Fig. 4C in VI). In addition, the $KCC2(-309/+42)^{Egr4mut}$ reporter was not anymore induced by BDNF (Fig. 4C in VI). The endogenous level of KCC2b mRNA was also rapidly upregulated by BDNF treatment (Fig. 2A in VI). Interestingly, while the KCC2b mRNA was upregulated,

the KCC2a mRNA expression slightly decreased after BDNF application (Fig. 2A in VI). The mRNA data were confirmed on a protein level (Fig. 1, 2B). The KCC2 protein upregulation was not observed in the mature neurons (Fig. 1A).

To sum, the above experiments demonstrate that a brief application of BDNF rapidly induces the ERK signalling pathway, increases Egr4 expression, and upregulates (via the $Egr4^{KCC2}$ site) the *SLC12A5-1b* promoter activity in immature dissociated neuronal cultures.

4.5.2 Regulation of the *SLC12A5-1b* promoter by Egr4: conclusions

Multiple *in vitro* experiments performed in N2a cells and in dissociated cultured neurons have shown that $Egr4^{KCC2}$ can regulate activity of the *SLC12A5-1b* promoter. Reducing $Egr4^{KCC2}$ function by decreasing Egr4 expression (using RNAi), by preventing Egr4 binding to $Egr4^{KCC2}$ (using DN-Egr4), or by mutating the $Egr4^{KCC2}$ site was able to downregulate the *SLC12A5-1b* promoter activity as well as the endogenous KCC2b expression *in vitro*.

Additional experiments are needed to address the role and relative importance of Egr4 in the *SLC12A5-1b* promoter regulation *in vivo*. An obvious approach would be to analyze whether KCC2b expression is reduced in Egr4 KO mice. Such mice have been generated by the group of Milbrandt (Tourtellotte *et al.*, 1999). The major phenotype reported for the Egr4 KO mice was infertility in males but not in females as a consequence of the impaired male germ cell maturation. This result is surprising, as *in vivo* Egr4 demonstrates mostly neural specific expression. Tourtellotte and coauthors have not reported any phenotypic abnormalities in the nervous system of the Egr4 KO mice. There are two possible explanations to this fact.

First, it has been shown previously that Egr1 is strongly (~9-fold) upregulated in the Egr4 KO mice (Tourtellotte *et al.*, 1999), thus compensation by Egr1 cannot be excluded. However, Egr1-Egr4 double KO mice have also failed to demonstrate any obvious CNS abnormalities; the major phenotype reported was just a complete infertility of these mice (Tourtellotte *et al.*, 2000). Unfortunately, no systematic behavioural analysis of Egr1-Egr4 KO mice has been performed to reveal putative mild brain abnormalities. In addition, compensation by two other Egr family members cannot be excluded.

Second, even in a case of the decreased KCC2b expression in the Egr4 KO mice, this downregulation might be insufficient to produce any obvious phenotypic abnormality. The maximum downregulation of the *SLC12A5-1b* promoter activity, achieved by mutating the $Egr4^{KCC2}$ site, was ~50% (Fig. 5B in IV); other methods resulted only in ~25-50% decrease (papers IV and VI). Thus, the KCC2b level in the Egr4 KO mice may still be ~50% of the wild type level. Is the ~50% downregulation of KCC2 expression level enough to be somehow reflected in the behaviour of these mice? Several KCC2-deficient mice models exist nowadays. Two complete KCC2 KO models have been generated by deleting either exon-4 (Vilen *et al.*, 2001; Tornberg *et al.*, 2005) or exon-5 (Hubner *et al.*, 2001) of the *SLC12A5* gene. Mice homozygous for the mutant allele die immediately after birth due to abolished respiration. However, no obvious phenotypic abnormalities have been detected in mice heterozygous for the null allele,

which retain 50% of the wild type KCC2 expression (Hubner *et al.*, 2001). Moreover, hypomorphic KCC2 mice retaining only ~30% of the wild type KCC2 level (Vilen *et al.*, 2001) are still viable and display no gross histological abnormalities. Surprisingly, adult mice doubly heterozygous for KCC2 null and hypomorphic alleles, expressing only ~15% of the wild type KCC2 level, display no gross histological abnormalities either (Tornberg *et al.*, 2005). Only a thorough behavioural analysis of these mice revealed some deviations from the normal behaviour: increased anxiety-like behaviour, higher susceptibility to pentylenetetrazole-induced seizures, impaired water maze learning, and reduced sensitivity to tactile and noxious thermal stimuli (Tornberg *et al.*, 2005). Of note, the KCC2 null and hypomorphic alleles affect expression of both KCC2a and KCC2b isoforms, while the Egr4 deficiency should only affect KCC2b expression. Thus, even in a case of a complete prevention of the KCC2 regulation by Egr4, the expected decrease of KCC2b expression seems not to be sufficient to produce any pronounced behavioural abnormality. A more conclusive strategy to study the role of Egr4 in the regulation of the *SLC12A5-1b* promoter *in vivo* would be to generate knock-in mice with the Egr4^{KCC2} site mutated/deleted and to measure the KCC2b expression level in these mice.

4.5.3 Regulation of the *SLC12A5-1b* promoter by USF1/2 transcription factors

E-box binding site (E-box^{KCC2}), highly conserved among mammalian *SLC12A5* gene orthologs, has been identified in the proximal *SLC12A5-1b* promoter (IV, V). Bioinformatical analysis predicted that multiple TFs are able to bind E-box^{KCC2}: USF1/USF2, the hypoxia inducible factor, PAS family members, N-myc, Clock/BMAL1, and NPAS2/ BMAL1. Many of these TFs are known to be expressed in CNS and thus could potentially regulate KCC2b expression. USF1/2 proteins were chosen to be characterized first for several reasons. Both USF1 and USF2 KO mice display a predisposition for spontaneous epileptic seizure activity (Sirito *et al.*, 1998) – the phenotype observed in the *SLC12A5* deficient mice. Moreover, several reports have demonstrated that USF1/2 factors are able to mediate activity-induced promoter regulation (Tabuchi *et al.*, 2002; Chen *et al.*, 2003; Steiger *et al.*, 2004). As discussed in the "Review of the literature", GABAergic (Ganguly *et al.*, 2001;Leitch *et al.*, 2005;Simat *et al.*, 2007), glutamatergic (Gulyas *et al.*, 2001; Sernagor *et al.*, 2003;Takayama & Inoue, 2006;Kanold & Shatz, 2006;Sipila *et al.*, 2009), and cholinergic neuronal activity (Liu *et al.*, 2006) may play an important role in the *SLC12A5* gene regulation. On the other hand, USF1/2 TFs may contribute to neuron-specific and mitogen-activated protein kinase (MAPK)-mediated regulation of gene expression (Park *et al*., 2008). Neuron-specificity and MAPK-regulation of the *SLC12A5-1b* promoter have been established in papers III and VI, respectively.

A possible role of the USF1/2 TFs in the *SLC12A5-1b* promoter regulation was assessed first in cell lines. Immunoblotting detected endogenous USF2 expression in C6 rat glioma cells, NIH3T3 fibroblasts, and mouse neuroblastoma N2a cells (Fig. 1A in V). USF2 expression in brain tissue was much stronger than in the cell lines.

EMSA was used to study further whether the endogenously expressed USF1/2 proteins are able to bind E-box^{KCC2}. A strong DNA-protein complex was detected after incubation of a 20-bp radioactively labelled probe bearing E-box^{KCC2} (Fig. 1B in V) with N2a nuclear extracts (Fig. 1C in V). Formation of the complex was effectively prevented by a 100-fold molar excess of unlabelled (cold) double stranded oligonucleotides bearing the intact E-box^{KCC2} site (Fig. 1C in V), but not by cold oligonucleotides bearing mutated E-box^{KCC2} (Fig. 1C in V). To confirm that USF1 and USF2 indeed participated in the DNA-protein complex formation, the nuclear extracts were preincubated with either USF1 or USF2 antibodies. Both USF1 and USF2 antibodies completely supershifted the DNA-protein complexes, while a control Egr4 antibody was ineffective (Fig. 1C in V). Similar results were obtained in NIH3T3 cells (Fig. 1D in V). In agreement with the lower USF2 expression level in NIH3T3 cells compared to N2a cells (Fig. 1A in V), DNA-protein complexes were weaker. Formation of the radioactively labelled complexes was efficiently competed by cold oligonucleotides containing intact but not mutant E-box^{KCC2} site (Fig. 1D in V). Similar to the situation in N2a cells, both USF1 and USF2 antibodies but not Egr4 antibody completely supershifted the DNA-protein complexes (Fig. 1D in V). Thus, EMSA experiments confirmed that endogenous USF1/2 proteins are able to bind the E-box^{KCC2} site. Gel shift experiments also showed that E-box^{KCC2} interacts preferentially with USF1/USF2 proteins, as USF1/2 antibodies supershifted completely the DNA-protein complexes. Of note, all TFs predicted previously by bioinformatics as potential candidates for binding the E-box^{KCC2} site (USF1, USF2, N-myc, CLOCK, NPAS2, hypoxia inducible factor, and BMAL1) are expressed in the N2a and NIH3T3 cell lines. Thus, USF1/2 proteins seem to have higher binding specificity to E-box^{KCC2} compared to other bHLH TFs in N2a and NIH3T3 cell lines.

To test whether USF1/2 TFs regulate the *SLC12A5-1b* promoter activity via the E-box^{KCC2} site, two reporter constructs were made. First construct [*KCC2b(0.6)*] contained a luciferase coding sequence under control of the ~0.6-kb *SLC12A5-1b* proximal promoter region, which bears the E-box^{KCC2} site. Second construct [*KCC2b(0.6)*$^{E\text{-}box\text{-}mut}$] was identical to the first except that E-box^{KCC2} was mutated. Luciferase assays with these constructs revealed that mutation of the E-box^{KCC2} site decreased the *SLC12A5-1b* proximal promoter activity by ~25% in N2a (Fig. 2A in V) and by ~35% in NIH3T3 (Fig. 2B in V) cells. These results were further confirmed by using A-USF, a dominant-negative mutant of USF1/2 TFs. A-USF forms heterodimers with USF1 and USF2, but these dimers are unable to bind E-box elements (Qyang *et al.*, 1999; Chen *et al.*, 2003). Overexpression of A-USF decreased the reporter activity of the *KCC2b(0.6)* construct both in N2a and NIH3T3 cells to the level of the *KCC2b(0.6)*$^{E\text{-}box\text{-}mut}$ construct. The overexpression had no effect on the reporter activity of the *KCC2b(0.6)*$^{E\text{-}box\text{-}mut}$ construct, in which the E-box^{KCC2} was mutated, thus excluding the possibility that A-USF acted indirectly on the *SLC12A5-1b* promoter. This experiment confirmed that E-box^{KCC2} is the only E-box site in the *SLC12A5-1b* proximal promoter that mediates regulation by USF1/USF2 TFs.

To provide evidence that USF1/2 TFs regulate the *SLC12A5-1b* promoter via binding E-box^{KCC2} also in primary neurons, ChIP using USF1 and USF2 antibodies was performed. ChIP demonstrated that USF1 and USF2 both interact with E-box^{KCC2} in div8 cultured rat cortical neurons (Fig. 3A in V). To examine whether USF1/2 TFs also regulate activity of the *SLC12A5-1b* promoter in primary neurons, luciferase assays with the *KCC2b(0.6)* and *KCC2b(0.6)*$^{E\text{-}box\text{-}mut}$ constructs were performed. Reporter activity of the *SLC12A5-1b* proximal promoter in cultured rat cortical neurons at div6 was downregulated significantly by mutation of the E-box^{KCC2} site (Fig. 3B in V). Interestingly, no difference between the reporter activities of the *KCC2b(0.6)* and *KCC2b(0.6)*$^{E\text{-}box\text{-}mut}$ constructs was detected in more matured cultured neurons at div10 (data not shown). This suggests that USF1 and USF2 factors might be more important in the developmental upregulation of the *SLC12A5-1b* promoter activity in immature than in mature neurons.

4.5.4 Regulation of the *SLC12A5-1b* promoter by USF1 and USF2: conclusions

The above experiments revealed that USF1 and USF2 TFs can bind E-box^{KCC2} and regulate the *SLC12A5-1b* promoter *in vitro* in cultured rat cortical neurons. Comparison of the *KCC2b(0.6)* and *KCC2b(0.6)*$^{E\text{-}box\text{-}mut}$ reporter activities suggested that the impact of the E-box^{KCC2} site on the promoter activity is ~30% in cell lines and in div6 cultured neurons.

Interestingly, mutation of the E-box^{KCC2} site had no effect in more matured neurons at div10. What could explain this? One possibility is that in more mature neurons E-box^{KCC2} is masked by certain TFs whose binding sites are located nearby. Such a situation has been described previously for the *$GABA_BR1b$* promoter, where E-box and CRE consensus binding sites are situated in a close proximity from each other (Steiger *et al.*, 2004). In that case USF1/2 complex is able to interact with the corresponding E-box only in the absence of CREB binding to the CRE site. To address this possibility for the *SLC12A5-1b* promoter one would use EMSA and gel shift to check whether TFs other than USF1/2 are also able to interact with the E-box^{KCC2} in matured neurons.

Another possible explanation could be that the USF1/2 complex is transcriptionally active in immature neurons even at basal conditions, but requires some additional activation in div10 neurons. This hypothesis is based on the fact that phosphorylation of USF1/2 is needed for its efficient binding to E-box (Cheung *et al.*, 1999; Galibert *et al.*, 2001; Xiao *et al.*, 2002; Chen *et al.*, 2003). The USF phosphorylation is known to be mediated by ERK1/2, p38, PKA, CaMKII, and CaMKIV in neurons (Chen *et al.*, 2003) as well as by p38, PKA, PKC, cdk1 in other cell types (Cheung *et al.*, 1999; Galibert *et al.*, 2001; Xiao *et al.*, 2002). Thus, analysis of the USF1 and USF2 phosphorylation status might help to unveil the functionality of the USF-E-box^{KCC2} pathway in regulation of the *SLC12A5-1b* promoter in div10 cultured neurons.

5 CONCLUSIONS

The major finding of the thesis is the discovery of the second KCC2 transcript (KCC2a). Up to recently, only one KCC2 isoform (KCC2b) was thought to exist, thus many authors studied the KCC2 expression using primers, probes, and antibodies detecting both isoforms simultaneously. This fact substantially entangled analysis of the mechanisms regulating the *SLC12A5* gene activity. Presented results demonstrate that KCC2a and KCC2b differ in their most N-terminal parts and that their expression is regulated by separate promoters, hence requiring a revision of the previous data.

Another important result of the thesis is the characterization of the spatio-temporal patterns of the KCC2a and KCC2b expression. To do that, isoform-specific antibodies, PCR primers, as well as *in situ* and RPA probes were exploited. Both isoforms were detected exclusively in CNS neurons and displayed similar levels of expression in embryonic and early postnatal development. Subsequently, KCC2a fraction in the total KCC2 expression decreased in most of CNS areas, constituting only ~10% in adult neurons. In cerebral cortex, the decline of the KCC2a/KCC2total ratio was a result of a dramatic upregulation of the KCC2b expression.

To elucidate mechanisms of the neuronal specificity and of *SLC12A5-1b* promoter activation during postnatal development, the corresponding genomic sequence was analyzed by bioinformatics. The neuron-restrictive silencing element in the intron-1b ($NRSE^{KCC2}$) was suggested as a candidate to explain the neuronal profile of KCC2 expression. Preliminary *in vitro* data confirmed this hypothesis; however subsequent *in vivo* experiments showed that this element is not sufficient for the KCC2 neuronal specificity. Additional experiments revealed that the upstream 1.4-kb fragment of the *SLC12A5-1b* promoter is enough for the neuronal specificity. Thus, yet to be defined mechanism (e.g. methylation) may act either alone or in synergy with $NRSE^{KCC2}$ to suppress the *SLC12A5-1b* promoter outside CNS neurons.

Two transcription factor binding sites important for *SLC12A5-1b* promoter activation have been characterized in the thesis. Mutation of either of them decreased activity of *SLC12A5-1b* promoter by ~35%. $E\text{-}box^{KCC2}$ site mediated the *SLC12A5-1b* upregulation via binding the upstream stimulatory factors 1 and 2 (USF1/2), while the $Egr4^{KCC2}$ site required specific interaction with early growth response 4 (Egr4) transcription factor. In addition, the data presented in the thesis suggested that the $Egr4^{KCC2}$ site might participate in the BDNF-induced ERK-mediated upregulation of KCC2b expression in early postnatal neurons.

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